University of Alberta

Effect of Beta-Amyloid Protein on Nicotinic Acetylcholine Receptor Function in Diagonal Band of Broca Neurons

by

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ABSTRACT

β-amyloid (Aβ), a 39-43 amino acid peptide deposited in the brains of Alzheimer's patients, disrupts synaptic function through mechanisms that are not fully understood. Nicotinic acetylcholine receptors (nAChRs) are potential targets for the actions of Aβ in the brain. This thesis examines 1) the role of nAChRs on basal forebrain neurons in Ca^{2+} signaling and excitatory neurotransmission and 2) if Aβ influences these functions via nAChRs.

Intracellular calcium levels ($[Ca^{2+}]_i$) in acutely dissociated rat basal forebrain neurons were measured with the ratiometric dye fura-2. Focal applications of nicotine, evoked increases of $[Ca^{2+}]_i$ mediated via Ca^{2+} entry via nAChRs and intracellular Ca^{2+} release. Nicotine-evoked $[Ca^{2+}]_i$ rises were inhibited by α-bungarotoxin, and dihydro-beta-erythroidine (DHβE). Nicotine-evoked increases were irreversibly potentiated by $Aβ_{1-42}$, while focal application of $Aβ_{1-42}$ alone did not alter $[Ca^{2+}]_i$. $Aβ_{1-42}$ also potentiated caffeine-mediated, but not KCl-evoked rises of $[Ca^{2+}]_i$. $Aβ_{1-42}$ potentiation of nicotine-mediated rises of $[Ca^{2+}]_i$ was blocked by either the SERCA inhibitor thapsigargin or CGP-37157, an inhibitor of the mitochondrial Na^+/Ca^{2+} exchanger. Thus $Aβ_{1-42}$ potentiation of repeated release of Ca^{2+} from intracellular stores by multiple nicotine challenges is due to overfilling of caffeine sensitive stores by mitochondria.

The effects of $Aβ_{1-42}$, nicotine and acetylcholine (ACh) on cholinergic basal forebrain neurons prelabeled with Cy3-IgG192 or identified by electrophysiological criteria were examined by recording miniature excitatory synaptic currents (mEPSCs) using whole-cell patch clamp recordings in brain slice preparations. In 54% of neurons, $Aβ_{1-42}$ significantly increased mEPSC frequency while in 32% of neurons, $Aβ_{1-42}$

significantly decreased mEPSC frequency. DHβE blocked $A\beta_{1-42}$ mediated increases of mEPSC frequency, but did not block $A\beta_{1-42}$ mediated decreases of mEPSC frequency. Nicotine or ACh in the presence of atropine, increased the frequency of mEPSCs in a dose-dependent manner. No changes in peak amplitude were observed in any treatments. These data suggest that in a subset of cholinergic neurons, $A\beta_{1-42}$ increases mEPSC frequency by activating pre-synaptic $\alpha4\beta2$ nAChRs, while another unknown pre-synaptic mechanism mediated the decrease of mEPSC frequency. As the regulation of $[Ca^{2+}]_i$ and synaptic transmission are important for cognitive functioning, these results may be important to understanding the pathophysiological mechanisms underlying Alzheimer's disease.

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Abbreviations

Aβ	β-amyloid peptide
ACh	acetylcholine
AChE	acetylcholinesterase
ACSF	artificial cerebrospinal fluid
AD	Alzheimer's Disease
AHP	afterhyperpolarization
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
APP	amyloid precursor protein
APV	(-) 2-amino-7-phosphonopentanoate
BHQ	di-(tert-butyl)-1,4-benzohydroquinone
αBTX	α-bungarotoxin
Ca^{2+}	calcium
$[Ca^{2+}]_i$	intracellular concentration of calcium
CGP-37157	7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one
ChAT	choline acetyltransferase
CNQX	cyano-7-nitroquinoxaline-2,3-dione
CREB	cAMP regulatory element binding protein
DBB	diagonal band of Broca
DHβE	dihydro-beta-erythroidine
EPSC	excitatory post-synaptic current
ER	endoplasmic reticulum
FAHP	fast afterhyperpolarization

GABA	γ-aminobutyric acid
InsR	inositol 1,4,5-phosphate receptor
IP_3	inositol 1,4,5-triphosphate
KS	Kolmogorov-Smirnov
LTP	long-term potentiation
NMDA	*N*-Methyl-D-Aspartic Acid
NO	nitric oxide
MAP2	mitogen activation protein kinase
mEPSCs	miniature spontaneous excitatory postsynaptic currents
PKC	protein kinase C
RyR	ryanodine receptor
SERCA	sarco-ER Ca^{2+} ATPase
SAHP	slow afterhyperpolarization
TG	thapsigargin
VGCC	voltage-gated calcium channel

General Introduction

Chapter 1

Overview

Alzheimer's disease (AD) is a devastating neurodegenerative disease that affects elderly individuals and is characterized by a progressive decline in memory and other cognitive functions such as language and perception (McKhann et al., 1984). The majority of AD occurs in a sporadic form, with an average age of onset of 70 years of age. Apolipoprotein E gene, which regulates the transport of cholesterol, has been identified as a susceptibility gene for the common late-onset form of AD (Poirier et al., 1993; Saunders et al., 1993). In a small subset of AD individuals, the onset of symptoms begin at approximately 50 years of age and these cases are associated with familial autosomal dominant mutations in transmembrane proteins regulated by genes located on chromosomes 21 (Amyloid Precursor Protein, (APP), 14 (Presenilin 1) and 1 (Presenilin 2)(Goate et al., 1991; Levylahad et al., 1995; Sherrington et al., 1995). Key neuropathological findings in individuals with AD include extracellular and neuritic (intracellular) plaques composed of β-amyloid peptide (Aβ), neurofibrillary tangles composed of abnormally phosphorylated tau protein, and selective loss of acetylcholine (ACh) synthesizing (cholinergic) neurons of the basal forebrain (Yankner, 1996; Mayeux and Sano, 1999). The loss of cholinergic neurons, which are one of the most vulnerable neuronal populations in AD, is associated with severe memory and learning deficits, which are the hallmarks of the clinical symptom complex of AD. The role of Aβ, a 39-43 amino acid peptide) in the pathogenesis of AD is controversial, i.e. whether it is a cause or consequence of the disease. However a large body of experimental evidence indicates that it may be a critical determinant of synaptic dysfunction and cell death that has been observed in many animal models of AD and the human condition (Hardy and

Allsop, 1991; Selkoe, 2002b). For example, Aβ has also been demonstrated to be neurotoxic to rat hippocampal and human cortical neurons (Mattson et al., 1993). Despite its wide-ranging effects, however, no definitive "receptor" has been identified for Aβ. Several candidate receptors have been proposed to mediate the biological actions of Aβ, including plasma membrane receptors for advanced glycation end products (RAGE), tumor necrosis factor receptor, and the p75 neurotrophin receptor (Rabizadeh et al., 1994; Barger et al., 1995; Yan et al., 1996). Aβ has been demonstrated to bind to nicotinic acetylcholine receptors (nAChRs) in nano- and picomolar concentrations and these receptors are important for mediating many cellular processes such as calcium (Ca^{2+}) signaling and neuromodulation. However, the interactions of Aβ with nAChRs are only beginning to be investigated and have not been studied in the basal forebrain, a region which is at the epicenter of the chemical pathology observed in AD (Castro and Albuquerque, 1995; Wang et al., 2000b; Wang et al., 2000a). **Therefore, the overall objective of my thesis was to 1) examine the role of nAChRs on basal forebrain neurons in Ca^{2+} signaling and neuromodulation 2) examine how Aβ influences nAChR mediated neuromodulation and Ca^{2+} signaling 3) examine the effects of Aβ on nAChR modulation of excitatory synaptic transmission in a cholinergic basal forebrain nucleus.**

I. Anatomy of the Basal Forebrain Cholinergic System

The basal forebrain nuclei are the major sources of cholinergic innervation to the brain in rats and humans (Mesulam et al., 1983a; Saper and Chelimsky, 1984). These nuclei are located ventral to the anterior horn of the lateral ventricle and the striatum and extend from the base of where the cerebral hemispheres unite and extend to the cerebral

peduncle. In coronal sections of rat brain, the anterior portion of the basal forebrain resembles an inverted "Y". The dorsal stalk of the Y is composed of the medial septum and the vertical limb of the diagonal band of Broca (DBB), while the horizontal arms of the Y comprise the horizontal limbs of the DBB (Harkmark et al., 1975). The most posterior sector of the basal forebrain is the nucleus basalis of Meynert, which can be further subdivided into anterior, intermediate, and posterior segments (Mesulam et al., 1983b; Mesulam et al., 1983a).

A. Neurochemical Characteristics of the DBB

DBB neurons are heterogeneous and contain many different neurotransmitters and neuropeptides. The two main populations of neurons in the DBB are the cholinergic and γ-aminobutyric acid (GABA) synthesizing cells (Brashear et al., 1986). There is also a local population of glutamatergic neurons (Sotty et al., 2003; Manseau et al., 2005). The cholinergic and GABAergic neurons can be divided into a population of large and a population of small diameter neurons. The large neurons are believed to be projection neurons, while the small neurons release neurotransmitters locally within the nucleus (interneurons).

1. Acetylcholine

Within the DBB, it is estimated that between 34-45% of neurons are cholinergic using staining methods for detecting either choline acetyltransferase (ChAT), which is an enzyme involved in ACh synthesis, or acetylcholinesterase, an enzyme that breaks down ACh in the synaptic cleft (Eckenstein and Sofroniew, 1983; Mesulam et al., 1983a). The morphology of these ChAT immunoreactive neurons can be divided into two types based upon their cell diameter (Milner, 1991). The first type is the magnocellular neurons,

which are 20-30 μM in diameter and are either elongated or round. They have an abundant cytoplasm with a small indented nucleus and may contain lamellar bodies and are the principal projection neurons of the DBB (Mesulam and Van Hoesen, 1976). The second population of neurons is the parvocellular (15-20 μM) neurons, which are round and do not have lamellar bodies. These neurons are responsible for local release of ACh (Metcalf et al., 1988).

The magnocellular neurons of the DBB provide cholinergic innervation to many CNS regions (Mesulam and Van Hoesen, 1976). Cholinergic DBB neurons project to the olfactory bulb, the hippocampus and to many areas of the neocortex (Mesulam et al., 1983a). They play an important role in a number of physiological functions including memory (Waite et al., 1994) and theta rhythm (Gaztelu and Buno, 1982) which shall be discussed more fully in later sections. In AD, it is the cholinergic projection neurons of the basal forebrain that preferentially undergo neurodegeneration (Whitehouse et al., 1982).

2. GABA

Immunohistochemical labeling for GABA and the GABA synthesizing enzyme L-glutamate decarboxylase in the basal forebrain identified a population of GABAergic neurons (Panula et al., 1984; Onteniente et al., 1986). This population of neurons was estimated to comprise between 34-54% of the neuronal population in the basal forebrain (Griffith, 1988; Kiss et al., 1990). The morphology of GABAergic neurons in the DBB is multiform and variable (Onteniente et al., 1986). When compared to other GABAergic neurons of the basal forebrain, GABAergic neurons of the DBB are larger, with a 20 to 30 μm diameter versus 10-20 μm in the lateral septum (Onteniente et al., 1986). The

aforementioned observations of the DBB GABAergic neurons suggest that this population of neurons is distinct from other neurons in the basal forebrain and may consist of mainly projection neurons (Onteniente et al., 1986). Based on anterograde and retrograde tracer studies, GABAergic neurons of the DBB have been identified to project to the neocortex and the hippocampus (Freund, 1989; Freund and Gulyas, 1991).

3. Glutamate

Recently, a population of glutamate releasing neurons was identified in the basal forebrain (Gritti et al., 1997; Manns et al., 2001). Based upon retrograde tracer analysis and single cell reverse transcription polymerase reaction for vesicular glutamate transporters, glutamatergic neurons are medium in size with an average diameter of 17 μm and consist of neurons that project to the neocortex and the entorhinal cortex (Sotty et al., 2003; Wu et al., 2003). There are also local glutamatergic interneurons that synapse onto both GABAergic and cholinergic neurons (Wu et al., 2003; Manseau et al., 2005).

4. Other Chemical Phenotypes within the DBB

Neurons in the DBB express other neuromodulatory compounds including galanin, nerve growth factor (NGF), nitric oxide (NO), and vasopressin. Galanin is a polypeptide which is co-localized in a subset of cholinergic neurons of the DBB that project to the hippocampus (Melander et al., 1985; Skofitsch and Jacobowitz, 1985; Senut et al., 1989). It has been shown that galanin inhibits the release of ACh onto hippocampal neurons via a presynaptic mechanism (Fisone et al., 1987). However, electrophysiological data show galanin to inhibit a suite of potassium conductances on cholinergic basal forebrain neurons resulting in hyperexcitabiliy of these cells (Jhamandas et al., 2002). NO synthase, an enzyme responsible for the synthesis of NO

has been found to colocalize with the ChAT and nerve growth factor receptor, however, further studies need to be done to examine the role of NO role in the DBB (Kitchener and Diamond, 1993).

B. Afferent and Efferent Connections and their Relation to Function

The DBB has many afferent and efferent connections in the brain, which are important for theta rhythm, arousal, learning and memory. The following sections will be divided into a section outlining the anatomical connectivity of the DBB and another describing the role of DBB in theta rhythm, learning and memory.

1. Efferent Connections of the DBB

Projections from the DBB extend to many areas of the brain. Anatomical studies utilizing anterograde and retrograde tracers have shown that the DBB projects to the hippocampus, olfactory bulb, neocortex, hypothalamus, amygdala, dorsal raphe, and locus coeruleus (Divac, 1975; Conrad and Pfaff, 1976a, 1976b).

Both cholinergic and GABAergic DBB neurons project to the hippocampus, olfactory bulb and neocortex and these projections have been studied in great detail (Mesulam et al., 1983a; Senut et al., 1989; Kiss et al., 1990; Freund and Gulyas, 1991). The neurons projecting to the hippocampus reside in the vertical and horizontal limbs of the DBB, while neurons projecting to the olfactory bulb and the neocortex mainly reside in the horizontal limb of the DBB (Mesulam et al., 1983a; Gaykema et al., 1990). Ultrastructural characterization of cholinergic DBB neurons has yielded more information about the synaptic connections of neurons projecting to the hippocampus and the neocortex. Cholinergic fibres projecting to the hippocampus have en passant axon collaterals that synapse with local somata and dendrites in the DBB (Henderson et al.,

2001). Electrophysiological experiments suggest that these collateral projections exert a modulatory effect on DBB neurons (Henderson et al., 2005). The main cholinergic fibres project to the hippocampus via the fornix and synapse onto hippocampal neurons (Nyakas et al., 1987). These hippocampal synapses terminate in the CA1 and CA3 regions onto pyramidal and granule neurons (Nyakas et al., 1987). GABAergic neurons of the DBB project along the fornix and synapse upon GABAergic neurons in the stratum oriens of the CA1 region of the hippocampus (Gulyas et al., 1990).

The cholinergic neurons of the DBB also project to the cingulate, visual, and olfactory cortices (Bigl et al., 1982; Luskin and Price, 1982; McKinney et al., 1983). GABAergic neurons of the DBB mainly project to the cingulate cortex, however, the projection to the visual cortex is sparse (Freund and Gulyas, 1991). The GABAergic terminals from DBB neurons form en passant synapses on GABAergic cell bodies and dendritic spines in all layers of the cortex (Freund and Gulyas, 1991).

The DBB projections that are important for cardiovascular regulation include projections to the supraoptic and paraventricular nuclei of the hypothalamus and the amygdala (Meibach and Siegel, 1977; Oka and Yoshida, 1985; Kovacs and Versteeg, 1993). DBB projections to the amygdala are also involved in memory and learning (Gallagher et al., 1977; Stock et al., 1981; Hostetter et al., 1987).

The DBB also projects to the dorsal raphe and the locus coeruleus which are areas with high concentrations of biogenic amines (Lee et al., 2005). The dorsal raphe nucleus contains somatostatin expressing neurons and receives a non-cholinergic, inhibitory innervation which is believed to be important in REM sleep (Kalen and Wiklund, 1989; Guzman-Marin et al., 2000). Little is known about the projection to the locus coeruleus,

except that a few identified neurons projecting from DBB to the locus coeruleus, also project to the dorsal raphe (Lee et al., 2005). The functional implications of this collateral branching connectivity to these two nuclei remain to be elucidated.

2. Afferent Connections to the DBB

The DBB receives inputs from many of the areas to which it projects. The reciprocal connectivity of these projections has implications for how the DBB functions in physiological processes. Anatomical studies have demonstrated that afferent input to the DBB originates from the cerebral cortex, hypothalamus, amygdala, the brainstem locus coeruleus and dorsal raphe.

Autoradiography and anterograde tracer techniques have shown glutamatergic and GABAergic neurons project from the hippocampus to the DBB (Freund, 1989; Carnes et al., 1990). Most of these projection neurons are GABAergic and form a reciprocal septohippocampal connection with GABAergic neurons of the DBB, however, some of the neurons also synapse onto the cholinergic neurons (Toth et al., 1993). There is also a minor glutamatergic projection from the hippocampus to the DBB (Carnes et al., 1990). The orbital, prefrontal, insular and olfactory cortices also provide some glutamatergic innervation to both the vertical and horizontal limbs of the DBB (Carnes et al., 1990; Zaborszky et al., 1997). These glutamate fibres cannot account for all the cortical projections to the DBB and the remaining fibres contributing to the cortical input to DBB are believed to be GABAergic.

The DBB receives significant input from all areas of the hypothalamus. Retrograde labeling has shown that there is significant glutamatergic innervation of the DBB from the medial and lateral aspects of the hypothalamus (Carnes et al., 1990). In

addition to glutamatergic innervation, vasopressin releasing neurons project to the DBB from the medial hypothalamus, the bed nucleus of the stria terminalis and the amygdala (Sofroniew, 1985; Caffe et al., 1989; Insel et al., 1994).

Catecholaminergic innervation of the DBB has been observed from the locus coeruleus and the dorsal raphe nuclei (Swanson and Cowan, 1979; Semba et al., 1988; Zaborszky and Cullinan, 1996). Although the precise chemical phenotype of these projections has not been definitively identified, they are most likely noradrenergic and serotonergic projections.

II. Physiological Functions of the DBB

Due to its extensive connectivity, the DBB is involved in a wide array of physiological functions in the central nervous system. The involvement of the DBB in theta rhythm, learning and memory are well characterized and important in the context of understanding how the loss of basal forebrain neurons causes cognitive dysfunction in the context of AD.

A. Theta Rhythm

Theta rhythm is a synchronized, low frequency (4-12 Hz) oscillation that is recorded in limbic structures (Leung et al., 1982). Initial studies associated theta rhythm with an arousal state. As theta is associated with a wide variety of physiological functions such as active exploratory sniffing (Forbes and Macrides, 1984), vibrissae movements (Semba and Komisaruk, 1984), eye movements during immobile visual exploration (Kemp and Kaada, 1975), and rapid eye movements in paradoxical sleep (Monmaur, 1981), it has been hypothesized that theta allows the hippocampus to

selectively receive inputs from sensory systems and voluntary motor systems to monitor performance in tasks (Vinogradova, 1995; Bland and Oddie, 2001; Buzsaki, 2002).

The involvement of the medial septum in the generation of theta rhythm was first observed in experiments in which lesioning of the DBB resulted in the loss of theta activity (Petsche et al., 1962). Electrophysiological experiments characterizing DBB neurons demonstrated that DBB neurons switch between firing bursts of actions potentials either in phase or out of phase with theta (Gaztelu and Buno, 1982; Sweeney et al., 1992). Bland and Colom (1993) hypothesized that theta frequency modulation is determined by the balance of septal GABAergic and cholinergic inputs to the hippocampus. As theta frequency rhythm decreases below 5 Hz, there is less inhibitory input from the DBB to the interneurons of the hippocampus, so that these interneurons are disinhibited. Consequently, the interneurons decrease the activity of neurons in phase with theta rhythm. As theta rhythm rises above 5 Hz, cholinergic-mediated EPSPs in the pyramidal neurons begin to dominate, resulting in a feed-forward increase in theta rhythm. The role of the DBB in theta suggests that these neurons may be important in maintaining an aroused state.

B. Learning and Memory

A central role for DBB in functions related to learning and memory is supported by several lines of evidence. Lesions to the DBB result in impairments in spatial learning, visual memory tasks and olfactory learning tasks (Hagan et al., 1988; Ridley et al., 1988; Paolini and McKenzie, 1993). Bilateral lesions of both GABAergic and cholinergic projection neurons in the DBB reduce the performance of rats in the Morris water maze suggesting that DBB projections are important in spatial memory (Hagan et

al., 1988). Subsequent studies in which cholinergic neurons were selectively lesioned demonstrated that although the loss of cholinergic neurons does not affect spatial learning when measured by the Morris water maze, rats with cholinergic lesions used different learning strategies to negotiate these tasks (Janis et al., 1998; Janisewicz and Baxter, 2003). The results of non-selective lesioning of the DBB and selective lesioning of cholinergic neurons showed that both GABAergic and cholinergic neurons play a role in spatial learning and memory.

The horizontal DBB connections to the olfactory bulb are involved in memory and learning as bilateral lesions of this region results in significant impairment in odor-reward associations and odor habituation (Paolini and McKenzie, 1993; Roman et al., 1993). These studies suggest that the horizontal DBB acts as a relay station between the olfactory bulb and the olfactory cortex. The DBB also plays a role in visual discrimination as excitotoxic lesions which reduce cholinergic activity in the cingulate cortex, an area where there is significant DBB projections, reduced performance in a visual discrimination test (Marston et al., 1994). These studies demonstrate that the DBB plays an important role in many different modes of learning and that loss of these projection neurons can significantly impair cognitive function.

C. DBB Neuronal Loss in AD

Post-mortem examination of brains from individuals with AD established a link between the preferential loss of basal forebrain cholinergic neurons and cognitive impairment (Whitehouse et al., 1982). The most severely affected regions in the basal forebrain were the medial septum which lost 50% of its neuronal population, and the DBB which lost 65% of its cholinergic neurons (Henke and Lang, 1983). Lesions of the

DBB in rats produce disruptions in spatial memory and these changes can be attributed to the loss of cholinergic neurons (Janis et al., 1998; Janisewicz and Baxter, 2003) In addition, to the depletion of cholinergic neurons, there is also accumulation of soluble Aβ, the deposition of insoluble amyloid plaques and the presence of neurofibrillary tangles in the basal forebrain (Saper et al., 1985). Given the importance of DBB cholinergic neurons in memory and learning, the significant degeneration of basal forebrain cholinergic neurons in AD may be responsible for the cognitive decline and that treatments designed to protect the loss of cholinergic cells may protect individuals against cognitive decline observed in AD.

III. Cellular Properties of DBB Neurons

A. Intrinsic Electrophysiological Properties

Initial examination of the electrophysiological properties of individual DBB neurons performed in guinea pigs identified 3 populations of neurons of this region (Griffith, 1988). The first neuronal cell type exhibited a slow afterhyperpolarization (AHP) of 600 ms with an amplitude of 10-20 mV which was attributed to a Ca^{2+} – activated potassium (K^+) conductance. Cells with the 600 ms AHP comprised 40% of the neurons recorded from and were called SAHP neurons. The second cell type identified exhibited a fast AHP (duration 5-50 ms) and comprised 53% of the cells recorded. The final cell type comprised the smallest population of neurons (7%) and fired in a burst pattern. Staining for AChE in cells that had been recorded from demonstrated that only SAHP neurons expressed AChE, suggesting that these were cholinergic neurons, while FAHP neurons were GABAergic. These findings were confirmed later on in slice preparations (Markram and Segal, 1990; Wu et al., 2000).

DBB neurons exhibit many voltage-activated and ligand-activated currents. A transient outward current (A-current) that was blocked by 4-aminopyridine and a suite of Ca^{2+} channels were described in acutely dissociated DBB cells (Griffith and Sim, 1990). Cholinergic neurons possessed both high voltage-activated and low voltage-activated Ca^{2+} channels, while non-cholinergic population only expressed high voltage-activated currents (Griffith and Sim, 1990). Further pharmacological analysis of cholinergic neurons demonstrated that N and L-type channels were the predominant population of Ca^{2+} channels, while the P/Q-type and T-type channels made up a small component of the population of Ca^{2+} channels (Murchison and Griffith, 1995, 1996; Easaw et al., 1999; Chin et al., 2002). These channels are modulated by many different ligands, suggesting that DBB neurons are influenced by a variety of neurotransmitters and neuromodulators (Chin and Jhamandas, 2002; Jhamandas and Mactavish, 2002).

B. Receptors Expressed on DBB Neurons

Slice recordings from the DBB demonstrated that there are many receptors systems involved in the regulation of neurons. Initial whole cell recordings demonstrated the presence of glutamate receptors on horizontal DBB neurons as perfusion of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) increased inward currents during voltage ramps, which were blocked by the AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Easaw et al., 1997). In addition, stimulation of the DBB evoked excitatory postsynaptic currents (EPSCs) that were blocked by CNQX, indicating the presence of a glutamatergic input onto DBB neurons. In the absence of magnesium, application of *N*-methyl-D-aspartate (NMDA) onto the slices also evoked an inward current that was inhibited by (-) 2-amino-7-phosphonopentanoate (APV). Further

examination of EPSCs showed the presence of a NMDA component on some cells (Easaw et al., 1997).

C. Intracellular Ca^{2+} stores

Ca^{2+} is an important intracellular ion, with many physiological functions ranging from involvement in release of neurotransmitters at synapses to regulation of neuronal death. Given the wide variety of functions that are influenced by intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), it is not surprising that it is very tightly regulated within individual neurons.

As discussed earlier, the basal forebrain neurons possess a suite of voltage gated Ca^{2+} channels and neurotransmitter gated channels that gate extracellular Ca^{2+} entry (Murchison and Griffith, 1995, 1996; Chin et al., 2002). The endoplasmic reticulum (ER) and the mitochondria are intracellular sources of Ca^{2+} release in the DBB (Murchison and Griffith, 1999, 2000).

The ER is a continuous endomembrane structure that extends throughout the neuron from the nuclear envelope to the distal dendrites (Martone et al., 1993). The ER accumulates intraluminal Ca^{2+} via the sarco-ER Ca^{2+} ATPase (SERCA) located on its membrane (Verkhratsky, 2005). Ca^{2+} is released from the ER by the activation of either inositol 1,4,5-phosphate receptor (InsR) or the ryanodine receptor (RyR), which are tetrameric Ca^{2+} channels that look remarkably similar under electron microscopy (Prentki et al., 1984; McPherson et al., 1991; Verkhratsky, 2005). Ca^{2+} is the endogenous ligand for RyRs, and in the DBB, these receptors amplify VGCC mediated $[Ca^{2+}]_i$ increases (Murchison and Griffith, 1999). A useful pharmacological tool used in the study of RyR is caffeine, which is a membrane soluble agonist of the RyR and an antagonist of the

InsR (Verkhratsky, 2005). At millimolar concentrations, caffeine is capable of raising $[Ca^{2+}]_i$, and depleting intracellular stores (Murchison and Griffith, 1999). The signal for release of Ca^{2+} from InsRs is transduced from the activation of a cell surface metabotropic receptor linked to the enzyme phospholipase C. Phospholipase C then cleaves phospholipids to generate inositol 1,4,5-phosphate (IP3), which diffuses to the ER and activates InsR (Berridge, 1993).

In addition to their role in energy production, mitochondria also play an important role in the regulation of Ca^{2+} in both pathophysiological and physiological states (White and Reynolds, 1996; Murchison and Griffith, 2000). Initial studies focusing on the role of mitochondria in excitotoxic neuronal death found that excess accumulation of Ca^{2+} in the mitochondria from influx of Ca^{2+} via glutamate receptors plays a key role in neuronal death (White and Reynolds, 1996; Stout et al., 1998). In DBB neurons, depolarization of the mitochondria has been shown to be capable of releasing Ca^{2+}, which suggests that mitochondria may play a role in the regulation of Ca^{2+} (Murchison and Griffith, 2000). Given the importance of these intracellular Ca^{2+} stores in regulating Ca^{2+} and apoptosis, they may be important targets for Aβ in mediating neurodegeneration in AD.

IV. Nicotinic Acetylcholine Receptor in the Central Nervous System

A. General Properties

Nicotinic acetylcholine receptors (nAChRs) are part of a superfamily of pentameric ligand gated channels that includes the serotonin, GABA, and glycine receptors. The properties of the nAChRs such as ligand affinity, opening probabilities, and cation permeability are dependent on subunit composition. The genes cloned for

neuronal nAChR subunits are divided into two subfamilies. The α subunit subfamily has 9 members (α2-α10), while the β subunit subfamily has 3 members (β2-β4).

The division of neuronal nAChR subunits into 2 subfamilies is based upon the homology of the neuronal α subfamily with the muscle α subunit, which both have consecutive cysteine residues at positions 192 and 193, while the β subunit subfamily does not have consecutive cysteine residues (Boulter et al., 1987). Two of the most commonly expressed nAChRs in the brain are the heteromeric α4β2 and homomeric α7 nAChRs. The subunits which form the α4β2 receptor along with the α3 subunit were initially discovered by low stringency DNA-DNA hybridization (Boulter et al., 1986; Boulter et al., 1987; Goldman et al., 1987). Electrophysiological studies performed on oocytes established that α4 and β2 subunits assemble to form functional nAChRs as cotransfection of these subunits yielded a greater current response to ACh than if the α4 subunit was expressed on its own (Boulter et al., 1987).

The initial pharmacological characterization of the α4β2 nAChR revealed that this receptor was not sensitive to blockade by α-bungarotoxin, a well established blocker of muscle nAChRs (Boulter et al., 1987). This suggests that there were additional nAChR subunits to be discovered as it was well established that there were α-bungarotoxin binding sites in the brain (Wang and Schmidt, 1976; Wang et al., 1978; Conti-Tronconi et al., 1985). Eventually, a new α subunit with a significantly different nucleotide sequence and splice sites from the α2-α6 subunits was cloned and termed the α7 subunit (Couturier et al., 1990; Schoepfer et al., 1990). Expression of the α7 subunit on its own in oocytes resulted in the formation of a nAChR channel with a rapidly desensitizing, α-bungarotoxin-sensitive inward current (Couturier et al., 1990).

The α4β2 and α7 channels display a significant degree of permeability to Ca^{2+}, an important second messenger in intracellular signal transduction pathways. The permeability of the α7 nAChR to Ca^{2+} is comparable to that of the NMDA glutamate receptor (Castro and Albuquerque, 1995). The α4β2 nAChR is not as permeable to Ca^{2+} as the α7 nAChR as the fraction of Ca^{2+} ions passing through α4β2 nAChRs is 2.6%, while the fraction of Ca^{2+} ions passing through α7 nAChR is 11% (Lax et al., 2002; Fucile et al., 2003). The significant Ca^{2+} permeability of nAChRs suggests that they play a role in Ca^{2+} regulation of DBB neurons and second messenger signaling.

B. NAChRs in the DBB

A recent report examining nAChRs in the DBB has shown that focal application of ACh onto medial septum/DBB neurons, elicits inward currents that can be blocked by mecamylamine, a α7 nAChR selective antagonist and dihydro-β-erythrodine, a non-α7 selective antagonist (Henderson et al., 2005). Of the neurons recorded from, a mecamylamine sensitive response was present in all cholinergic neurons examined and 10% of GABAergic neurons. However, 50% of GABAergic neurons displayed non-α7 nicotinic responses (Henderson et al., 2005). This data should be interpreted with caution as mecamylamine is not a pure α7 nAChR antagonist as it blocks both α7 and αβ heteromeric nAChRs in the micromolar range (Chavez-Noriega et al., 1997). This leaves open the possibility that other nAChRs could be expressed on cholinergic neurons. This data suggest that the nAChR receptor system plays an important role in the modulation of neuronal activity in the cholinergic basal forebrain system.

C. Physiological Functions of nAChRs in the Central Nervous System

1. Modulation of Transmitter Release

NAChRs play an important role in modulation of neurotransmitter release in the brain. Dopamine release from substantia nigra nerve terminals in the striatum was one of the first neurotransmitter systems found to be modulated by nAChRs (Westfall, 1974; Rapier et al., 1990). Initially, when brain slices containing the striatum were perfused with nicotine, an increase in dopamine release was observed which was attributed to activation of presynaptic nAChRs (Westfall, 1974). Pharmacological and molecular characterization of dopaminergic synaptic terminals established that activation of presynaptic $\alpha4\beta2$ nAChRs increases dopamine release in the striatum (Rapier et al., 1990; Sacaan et al., 1995; Sharples et al., 2000; Zoli et al., 2002). The first electrophysiological evidence for presynaptic modulation of neurotransmitter release was found in the medial habenula nucleus of the hypothalamus (McGehee et al., 1995). Activation of $\alpha7$ nAChRs was found to enhance glutamate release in a Ca^{2+} dependent manner (McGehee et al., 1995).

Neurons in areas involved in learning and memory such as the cortex and hippocampus are also modulated by nAChRs. In cortical interneurons, $\alpha4\beta2$ and $\alpha7$ nAChRs mediate both post-synaptic and pre-synaptic responses (Alkondon et al., 2000). Additionally, there are presynaptic $\alpha4\beta2$ nAChRs that increase the release of GABA onto cortical interneurons (Alkondon et al., 2000). In the hippocampus, $\alpha4\beta2$ and $\alpha7$ nAChRs are found to mediate postsynaptic currents in CA1 pyramidal neurons and interneurons, however, $\alpha7$ nAChRs are also found to increase presynaptic release of GABA (Alkondon and Albuquerque, 1993; Alkondon et al., 1997).

2. NAChRs and Second Messengers

Given that nAChRs have high permeability to Ca^{2+}, studies have established these receptors to be involved in signal transduction pathways crucial to cell survival. In pheochromocytoma cells, activation of nAChRs depolarizes the cell resulting in Ca^{2+} influx that activates Protein kinase C (PKC). PKC then phosphorylates Raf1, a mitogen activated kinase kinase kinase, starting a cascade that leads to the eventual phosphorylation of CREB which then activates gene transcription (Tang et al., 1998). In the prefrontal cortex, $\alpha4\beta2$ nAChR activation leads to activation of intracellular Ca^{2+} stores, which then activates PKC, leading to increased dopamine release through the dopamine transporter (Drew and Werling, 2001). Activation of $\alpha7$ nAChRs has been shown to cause Ca^{2+} entry via voltage gated Ca^{2+} channels, which leads to activation of intracellular Ca^{2+} stores via activation of InsRs (Dajas-Bailador et al., 2002). Release of Ca^{2+} from intracellular stores has been shown to activate the extracellular signal receptor kinase pathway (ERK1/2) which is important in learning and memory (Dajas-Bailador et al., 2002; Tomizawa and Casida, 2002). There are additional pathways to be discussed below.

3. Age-Dependent Alteration of nAChRs

In both rats and humans, nAChRs are present during the early moments of life. In rats, the $\alpha2$, $\alpha3$, $\alpha4$, $\alpha5$, $\alpha6$, $\beta2$ and $\beta4$ subunits are localized in the diencephalon, brainstem, spinal cord and telencephalon (Gotti and Clementi, 2004). However as the individual subunits need to combine with other subunits in order to form receptors, variations in the temporal expression of the subunits also varies the expression of functional nAChRs, for example, the $\beta4$ subunit precedes the $\alpha3$ subunit by 2 days,

which combine to form α3β4 nAChRs (Zoli et al., 1995). In the human brain, nAChR expression starts at 12 weeks of gestation and reaches an apex at 27 weeks of gestation. During the perinatal period and early infancy, nAChR concentration decreases in all brain areas except for the cerebellum, where concentrations remain constant. Interestingly, the highest concentration of nAChRs during fetal development was observed in the nucleus basalis of Meynert, a basal forebrain nucleus (Cairns and Wonnacott, 1988).

During ageing, nAChR binding sights decrease in both rats and humans. Both α7 and α4 subunit mRNA and protein levels were lower in aged humans (60-90 years of age) in the DBB, medial septum, and frontoparietal cortex which may contribute to cognitive decline (Tohgi et al., 1998). Declines in nAChR receptor expression has also been observed in rats, however, the level of nAChR loss is not associated with decreased performance in learning and memory tasks (Smith et al., 1995).

4. NAChRs and AD

NAChR expression in certain areas of AD brains is significantly reduced when compared to age-matched controls (Flynn and Mash, 1986). Removal of cholinergic input in rats by selectively lesioning cholinergic neurons in the basal forebrain leads to decreased performance in learning and memory task (Steckler et al., 1995). Furthermore, nicotinic agonist treatment improves performance in memory and learning tasks (Levin and Simon, 1998).

Studies measuring nicotinic radioligand binding comparing age-matched control brains with AD brains have established that there is a significant decrease in nAChR binding sites (Whitehouse et al., 1988; Warpman and Nordberg, 1995). These decreases in nAChR have been localized to mainly those areas of the brain associated with

cognition such as the frontal cortex, temporal cortex, and the hippocampus (Rinne et al., 1991; Warpman and Nordberg, 1995). Immunohistochemical examination of the subtypes of nAChRs frontal cortex revealed a 30% decrease in α4β2 and α7 nAChR neurons (Wevers et al., 1999). In the temporal cortex, α4β2 nAChR binding sites and α4 subunit expression were decreased, while there was no change in α3 and α7 subunit expression (Martin-Ruiz et al., 1999). In the hippocampus, there was a decrease in nAChR binding sites in the dentate granular layer, the presubiculum, and the parahippocampal gyrus, however, no changes in nAChR binding sites were observed in the CA1 or CA3 layers (Perry et al., 1995). The loss of specific nAChR binding sites in brain regions involved in memory and learning may play an important role in mediating the cognitive impairment observed in AD.

V. General Properties of Amyloid

Aβ, a 39-43 amino acid peptide, which was isolated from amyloid plaques, plays an important role in the AD pathophysiological process (Glenner and Wong, 1984; Masters et al., 1985a; Masters et al., 1985b). Initial studies of AD individuals detected no correlation between dementia and insoluble Aβ load, however, when both soluble and insoluble forms of Aβ were measured, the Aβ load in these areas correlated with the extent of cognitive decline (Terry, 1999; Naslund et al., 2000).

The amyloid hypothesis postulates that an increase of Aβ production is the primary pathophysiological mediator of AD (Selkoe, 2001). Three lines of evidence support the basis for this hypothesis. The deposition of Aβ in the brains of AD individuals is a major pathological finding. Secondly, application of Aβ onto a variety of animal and human neuronal preparations is neurotoxic (Mattson et al., 1992; Yan et al.,

1996; Nakagawa et al., 2000; Jhamandas and MacTavish, 2004). Finally, mutations associated with early onset AD increase Aβ production (Citron et al., 1992; Borchelt et al., 1996). These mutations are located either on proteolytic processing sites of APP, which is the precursor of Aβ or on the genes of proteins responsible for APP cleavage (Chartierharlin et al., 1991; Stgeorgehyslop et al., 1992). Individuals afflicted with Down's syndrome, who have an extra chromosome 21, which is the chromosome that APP is located on, exhibit severe mental retardation and developmental delay along with significant deposition of Aβ in their brains (Masters et al., 1985c).

A. Aβ production

Aβ is a cleavage product of a larger precursor protein, the APP (Kang et al., 1987; Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988). There are 8 known isoforms of the APP gene that are formed by differential splicing, however the major isoforms of APP are 695, 751 and 770 residues in length (Sandbrink et al., 1996). The 751 and 770 residue form of APP are different from the 695 residue form in that they have a Kunitz-type serine protease inhibitor domain (Kang et al., 1987; Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988).

APP has multiple cleavage sites and is processed either by a non-amyloidogenic or amyloidogenic pathway (Figure 1)(Selkoe, 2001). α-Secretase cleaves APP to generate a long fragment termed αAPP_s and an 83 amino acid carboxy-terminal peptide (Esch et al., 1990; Sisodia et al., 1990). Proteolytic cleavage of APP by α-secretase is the first step of non-amyloidogenic cleavage as it precludes the formation of Aβ (Buxbaum et al., 1998; Lammich et al., 1999). If APP is not processed by α-secretase, APP is cleaved by an enzyme termed β-secretase to generate βAPP_s, a fragment that is slightly

smaller than αAPP_s and a 99 amino acid carboxy-terminal fragment that begins at residue 1 of the Aβ protein (Vassar et al., 1999; Selkoe, 2001). The carboxy-terminal fragments produced by α- and β-secretase cleavage are subsequently cleaved by γ-secretase. γ-Secretase cleavage of the 83 amino acid COOH-terminal fragment yields a NH_2-terminal fragment terminal peptide termed p3, while cleavage of the 99 amino acid carboxy-terminal fragment yields the 39 to 43 amino acid forms of Aβ (Selkoe, 2001). The proteolytic enzyme responsible for γ-secretase activity is composed of 5 proteins, which are termed presenilin-1, presenilin-2, alph1, nicastrin and pen-2 that form an intramembrane complex to facilitate carboxy-terminal cleavage (Selkoe, 2002a).

B. Conformation of Aβ

After Aβ is secreted, it forms different conformations which determine its effects on neurons (Pike et al., 1993; Lambert et al., 1998; Walsh et al., 2002). The simplest forms of Aβ are the soluble monomeric and small oligomeric forms that are found in the cerebrospinal fluid (Hilbich et al., 1991; Seubert et al., 1992). The small oligomeric forms have been shown to inhibit long term potentiation, while the monomeric forms exhibited no effect (Walsh et al., 2002). In addition, small oligomeric forms are significantly more neurotoxic than either fibrils or monomers and the toxicity is specific to the aggregation of Aβ (Pike et al., 1993; Lambert et al., 1998; Dahlgren et al., 2002). Over time, oligomeric forms of Aβ eventually aggregate into non-soluble fibrils that are in equilibrium with soluble forms of Aβ. These are the major constituent of neuritic plaques (Hilbich et al., 1991). However, prior to the formation of fibrils, a transient proto-fibril conformation is observed (Harper et al., 1997; Walsh et al., 1997). The proto-fibril is 6-10 nm in diameter and between 30 and 500 nm in length, while fibrils

exhibit a beaded appearance, with a maximal diameter that is larger than the proto-fibril and extend for over 1 μM (Harper et al., 1997; Walsh et al., 1997). $A\beta_{1-42}$ is significantly more fibrilogenic than $A\beta_{1-40}$ (Harper et al., 1997). The fibrillar form of Aβ is neurotoxic, although it is significantly less neurotoxic than the soluble oligomeric forms (Dahlgren et al., 2002). The work on the different conformations of Aβ highlights the importance of soluble conformations of Aβ in mediating neuronal effects.

C. Effects of Aβ on neurons

Aβ has been shown to disrupt neuronal function and mediate neurotoxicity in many neuronal systems, however, its definitive role and receptor that mediates its actions remains unknown. Several pathways have been proposed to mediate Aβ neurotoxicity. One hypothesis is that Aβ mediates neurotoxicity by causing oxidative stress on neurons (Behl et al., 1994). Generation of free radicals results neuronal damage and activation of a pro-inflammatory transcription factor NF-kappa-B, which is believed to further contribute to neuronal death (Kaltschmidt et al., 1997). Another possible mediator of Aβ mediated neurotoxicity is the disruption of Ca^{2+} homeostasis (Mattson et al., 1992). The exact mechanism whereby Aβ affects Ca^{2+} homeostasis is unknown and many theories have been suggested. Evidence for Aβ peptides forming plasma membrane channels that pass Ca^{2+} ions have been observed in lipid bilayers, however there is no convincing physiological data in neurons demonstrating the passage of Ca^{2+} ions through these channels (Arispe et al., 1993; Rhee et al., 1998). Aggregated Aβ inhibits Ca^{2+} current through voltage-gated Ca^{2+} channels, while unaggregated Aβ inhibits this current (Ramsden et al., 2002). Aβ has been shown to disrupt the functioning of the ER, an important regulator of Ca^{2+} and results in the activation of caspases, the proteolytic

cascade which leads to apoptosis (Nakagawa et al., 2000). Aβ has also been shown in slice preparations to enhance glutamate release, which would lead to increased Ca^{2+} entry into neurons via glutamatergic receptors (Kar, 2005). *These studies support the hypothesis that Aβ disruption of Ca^{2+} homeostasis is an important step in mediating neurodegeneration.*

D. Aβ and nAChRs

A major conceptual advance in the role of the nAChR in neurodegeneration was the discovery that both the 40 and 42 amino acid forms of the Aβ protein could bind to nAChRs (Wang et al., 2000b; Wang et al., 2000a). Further studies have shown that Aβ is capable of functional modulation of the nAChR (Liu et al., 2001; Pettit et al., 2001; Dineley et al., 2002; Fu and Jhamandas, 2003; Grassi et al., 2003). In their original studies on Aβ-nAChR binding, Wang et al. (2000a) showed that Aβ were capable of competitively displacing α7 nAChR selective antagonists from synaptic membranes produced from guinea pig and rat cortex and hippocampus with picomolar affinity. Aβ was also found to be able to bind to α4β2 nAChRs with nanomolar affinity (Wang et al., 2000a). Additional evidence for the Aβ interaction with nAChR was obtained from co-immunoprecipitation data (Wang et al., 2000b). Aβ binding nAChR could potentially activate apoptotic pathways and the Aβ-nAChR complex may seed the development of amyloid plaques (Wang et al., 2000b). The nAChR-Aβ binding studies have been very interesting, however, it is problematic that other laboratories have had difficulty reproducing the original findings of Wang and co-workers (2000a, b).

Electrophysiological experiments examining the effect of Aβ on nAChR in the CNS have focused on two subtypes of nAChR, the homomeric α7 nAChR and the

heteromeric α4β2 nAChR. Initially, studies showed that in CA1 stratum radiatum interneurons, 100 nM $A\beta_{1-42}$ blocked α7 nAChR responses (Liu et al., 2001; Pettit et al., 2001; Grassi et al., 2003). Studies of the Aβ interaction with α7 nAChRs in *Xenopus* oocytes expression systems suggest that $A\beta_{1-42}$ weakly activates nAChRs resulting in desensitization of the α7 nAChRs (Dineley et al., 2002; Grassi et al., 2003). Dougherty et al. (2003) examined the effect of Aβ on presynaptic terminals and found that Aβ antagonizes homomeric α7 nAChR at picomolar concentrations by blocking agonist binding.

Further examination of Aβ actions at a cellular level also revealed that it has effects on α4 nAChRs (Fu and Jhamandas, 2003; Wu et al., 2004). In basal forebrain neurons, $A\beta_{1-42}$ activated α4β2 nAChRs in a dose dependent manner and this activation lead to depolarization of these neurons (Fu and Jhamandas, 2003). Another study examining the effect of Aβ on α4β2 nAChR expressed in a cell line revealed that Aβ blocks α4β2 nAChR in a non-competitive manner, however, when the cell line was transfected with functional α7 nAChRs, $A\beta_{1-42}$ was unable to block these receptors (Wu et al., 2004). Given the conflicting data with regards to $A\beta_{1-42}$ and nAChRs interactions, two issues remained to be resolved. Firstly, the nAChR subtype that Aβ interacts with is unclear and secondly, whether Aβ acts as an agonist or antagonist at such receptors remains to be resolved.

The consequences of these Aβ actions on nAChR interactions are undoubtedly wide-ranging but from a pathophysiological point of view, the effects of such interactions on Ca^{2+} homeostasis in the cell are perhaps the most intriguing. A potential consequence of the Aβ-nAChR interaction would be the excessive entry of Ca^{2+} into presynaptic

terminals resulting in inappropriate desensitization of nAChR and activation of second messenger pathways that may lead to neurodegeneration (Dougherty et al., 2003). Alternately, the interaction of Aβ and the nAChR may lead to internalization of the receptor and $A\beta_{1-42}$, where Aβ may mediate additional effects (Nagele et al., 2002).

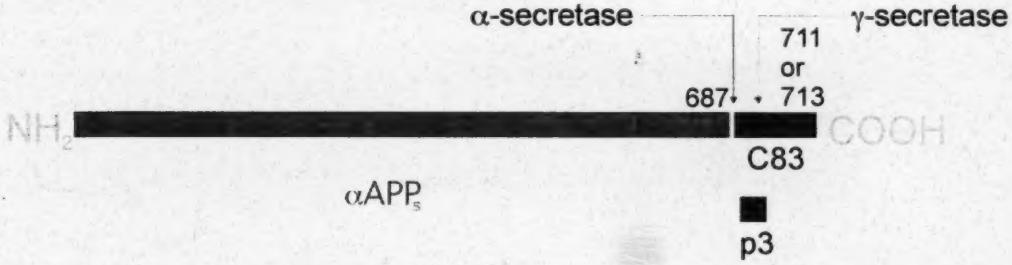
Activation of the α7 nAChR also activates second messenger systems important in neuroprotection. The effect of Aβ has been examined on the Janus kinase 2 (JAK2) and the mitogen activation protein kinase (MAP2) pathways, which are two neuroprotective pathways that the α7 nAChR activates (Dineley et al., 2001; Shaw et al., 2002). Activation of the JAK2 pathway results in the JAK2 enzyme phosphorylating itself and activating downstream mediators phosphatidyl inositol and akt to prevent activation of apoptotic pathways (Shaw et al., 2002). It was found that α7 nAChR mediated neuroprotection via this pathway in cultured neurons was inhibited by $A\beta_{1-42}$ (Shaw et al., 2002). Another possible mechanism via which $A\beta_{1-42}$ mediates its effects is via excessive activation of α7 nAChR, which would down-regulate the MAPK2 pathway and reduce the phosphorylation of its downstream target, the cAMP regulatory element binding protein (CREB). This cascade may lead to derangement of the signaling pathway (Dineley et al., 2001). These data demonstrate the importance of second messenger systems mediated by nAChRs and the ability of Aβ to modulate these systems. *As Aβ has been implicated to both disrupt synaptic function and disturb Ca^{2+} homeostasis, the studies on nAChR-Aβ interactions would shed light on the effect of Aβ on two important cellular functions; the tight regulation of $[Ca^{2+}]_i$ and synaptic function.*

VI. Objectives and Rationale

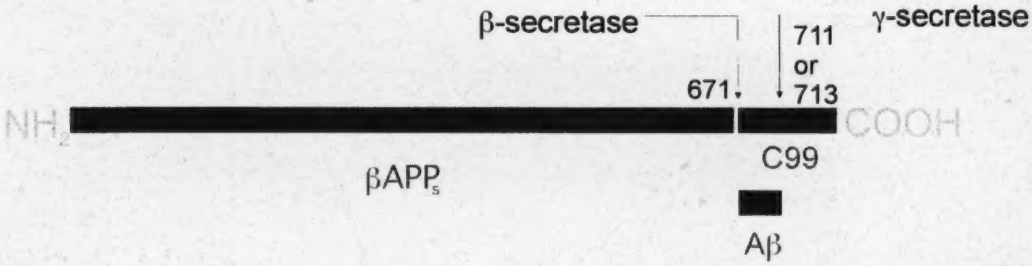
Given the recent data showing that Aβ binds to nAChRs, I examined the effect of Aβ on basal forebrain neurons in two ways. In the first part of my thesis, I examined nAChR mediated Ca^{2+} rises in acutely dissociated DBB neurons using the fluorescent ratiometric Ca^{2+} dye fura-2 and investigated the mechanisms whereby Aβ may modulate $[Ca^{2+}]_i$. In the second part of my thesis, I examined the actions of Aβ on nAChR modulation of excitatory synaptic neurotransmission in cholinergic DBB neurons using brain slices, an experimental preparation where the neuronal connectivity is intact.

A. APP 770

B. Non-amyloidogenic pathway



C. Amyloidogenic pathway



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Chapter 2

β-Amyloid Enhances Intracellular Calcium Rises Mediated by Intracellular Calcium Stores and Nicotinic Receptors in Acutely Dissociated Rat Basal Forebrain Neurons

INTRODUCTION

Alzheimer's disease (AD) is a devastating neurodegenerative disease that affects elderly patients and is characterized by a progressive decline in memory and other cognitive functions such as language and perception (McKhann et al., 1984). Key neuropathological findings in patients with AD include extracellular neuritic plaques composed of β-amyloid peptide (Aβ), neurofibrillary tangles composed of abnormally phosphorylated tau protein, and loss of cholinergic neurons of the basal forebrain (Yankner, 1996; Mayeux and Sano, 1999). Aβ, a 39-43 amino acid peptide, plays an important role in the AD pathophysiological process as total Aβ content in the brain correlates with cognitive decline (Naslund et al., 2000). Although the precise mechanism of Aβ toxicity has not been established, Aβ and other peptide fragments derived from amyloid precursor protein may destabilize intracellular calcium (Ca^{2+}) homeostasis via modulation of specific ion channels (Mattson et al., 1992; Fraser et al., 1997). An intriguiging class of candidate receptors for Aβ-mediated actions is the neuronal nicotinic acetylcholine receptors (nAChRs), a family of ligand-gated cation permeable channels with multiple subtypes that are widely distributed in the human brain. nAChRs are involved in a number of physiological and behavioral processes and post-mortem studies of AD individuals demonstrate that reduced numbers of nAChRs in brain regions are associated with AD (Nordberg and Winblad, 1986; Whitehouse et al., 1988; Paterson and Nordberg, 2000).

$A\beta_{1-42}$ binds to α7 and α4β2 subtypes of nAChRs with high affinity in the picomolar and nanomolar range respectively (Wang et al., 2000b; Wang et al., 2000a). In the rat basal forebrain, Aβ directly activates α4β2 or α3 postsynaptic nAChRs in the

diagonal band of Broca (DBB), a basal forebrain nucleus (Fu and Jhamandas, 2003). Based on previous data this Aβ-non-α7 nAChR interaction appears to occur on cholinergic rather than GABAergic basal forebrain neurons (Jhamandas et al., 2001).

Interactions of amyloid peptides with cholinergic neurons are considered to be a basis for the modified cholinergic tone observed in AD (Auld et al., 2002). Both α7 and non-α7 nAChRs are abundantly located within the rat basal forebrain cholinergic system (Dominguez del Toro et al., 1994; Rogers et al., 1998). Although the exact functional consequences of Aβ interaction with nAChRs remain unknown, nAChRs regulate many neuronal processes. They play an important role in neurotransmission, synaptic modulation, and second messenger signaling via intracellular Ca^{2+} stores (Tsuneki et al., 2000; Dajas-Bailador et al., 2002; Henderson et al., 2005). Thus, Aβ disruption of synaptic transmission and Ca^{2+} homeostasis could be mediated via an interaction with nAChRs (Mattson et al., 1992; Kamenetz et al., 2003).

The main sources of intracellular Ca^{2+} stores in the DBB are the endoplasmic reticulum (ER) and the mitochondria (Murchison and Griffith, 2000). These stores have been shown to be important in both the release and buffering of Ca^{2+} (Murchison and Griffith, 2000). Accumulation of Ca^{2+} in the mitochondria and endoplasmic reticulum has been associated with apoptotic cell death in pathophysiological conditions and $A\beta_{1-42}$ has been shown to mediate apoptosis via an ER-dependent pathway (White and Reynolds, 1997; Nakagawa et al., 2000). Thus examining Ca^{2+} regulation in these organelles is important to understanding Aβ neurotoxicity.

I examined the effect of $A\beta_{1-42}$ modulation of nAChRs mediated Ca^{2+} rises and the associated intracellular Ca^{2+} stores by performing ratiometric Ca^{2+} imaging in acutely

dissociated DBB neurons. nAChR activation was demonstrated to be associated with activation of intracellular Ca^{2+} release. I also demonstrated that $A\beta_{1-42}$ modulates the repeated activation of nAChRs by facilitating the release of Ca^{2+} from intracellular Ca^{2+} stores through overfilling of thapsigargin-sensitive stores.

MATERIALS AND METHODS

Dissociation Procedures

Ratiometric fura-2 imaging was performed on acutely dissociated rat DBB neurons. The protocol for preparation of tissue was reviewed and approved by the University of Alberta Health Sciences Laboratory Animal Services (Protocol number 154/04/05). Details of the procedure for acute dissociation of neurons from the DBB have been previously described (Chin et al., 2002). Briefly, brains were quickly removed from decapitated male Sprague Dawley rats (21-25 day postnatal) and placed in cold artificial cerebrospinal fluid (ACSF) that contained (in mM) 140 NaCl, 2.5 KCl, 1.4 $CaCl_2$, 5 $MgCl_2$, 10 HEPES, and 33 D-glucose (pH 7.4). Brain slices (350 μm thick) were cut on a vibratome, and the area containing the horizontal and vertical limbs of DBB was dissected out. Acutely dissociated neurons were prepared by the enzymatic treatment of slices with trypsin (0.65 mg/ml) at 30 °C for 16 min, followed by mechanical trituration for dispersion of individual cells. Cells were then plated on poly-L-lysine (0.005% wt/vol)-coated cover slips and viewed under an inverted microscope (Zeiss Axiovert 35). Neurons were identified based upon previously established visual criteria (Chin et al., 2002). Basal forebrain neurons could be differentiated from glia based upon the presence of truncated dendrites. In addition, neurons were larger than glia. All solutions were kept oxygenated by continuous bubbling with 100% oxygen.

Calcium Imaging

Neurons were incubated with 10 μM fura-2 AM (Molecular Probes, Eugene, Oregon, USA) in 1 ml of oxygenated ACSF for 20 min and perfused for 20 min prior to imaging. All experiments were performed at room temperature (20-22 °C). Measurements were collected every 8 seconds for 2 minutes to establish a baseline. During focal application of drugs, the image collection rate was increased to 1 Hz for 15 seconds and then returned to its baseline of rate of 1 image every 8 seconds. Fura-2 loaded neurons were exposed to 340 and 380 nm light emitted by a Polychrome IV monochromator (T.I.L.L. Photonics GMBH, Pleasanton, CA, USA) controlled by Metafluor 5.03 software (Universal Imaging Corporation, Downingtown, PA, USA) and emitted fluorescence was collected by a CoolSnap HQ CCD camera (Roper Scientific, Duluth, GA, USA). A three-point calibration was performed on acutely dissociated cells loaded with 100 μM fura-2 and EGTA/Ca^{2+} buffered solutions via patch pipette with a final concentration of 0, 224 nM and 5 mM of Ca^{2+} (Grynkiewicz et al., 1985). $[Ca^{2+}]_i$ for the pipette solutions was determined with MaxChelator 2.32 (Stanford, Chris Patton).

The values obtained from the calibrations were used to convert background subtracted experimental fluorescent intensity ratios into $[Ca^{2+}]$ over the physiological range of $[Ca^{2+}]_i$. using the following equation: $[Ca^{2+}]_i = K_d(R-R_{min})/(R_{max}-R)$, where K_d is the dissociation constant for fura-2, R_{min} is the background subtracted 340/380 ratio when $[Ca^{2+}]_i$ was 0, R_{max} is the background subtracted 340/380 ratio when $[Ca^{2+}]_i$ was 5 mM and R is the experimentally measured background subtracted 340/380 ratio (Grynkiewicz et al., 1985).

Immunohistochemistry

DBB cells were chemically and mechanically isolated from 14-21 day old Sprague Dawley rats as described above. The DBB cell suspension was centrifuged for 5 minutes at 500 rpm onto glass slides. Cells were not fixed. Cells were first blocked with 1% bovine serum albumin (Sigma, Oakville, CA) for 1 hour and then incubated overnight with primary rabbit antibodies for vesicular choline acetyltransferase (vChAT: 1:2500 dilution) α4 (1:250 dilution) or α7 (1:500 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, USA) and then washed 3 times in 0.1 M phosphate–buffered saline (PBS), pH 7.2. Cells were then incubated with biotinylated goat anti-rabbit antibodies for 1.5 hours and washed again with PBS. Next, cells were incubated with the fluorescent dye Alexa 546 streptavidin to yield red fluorescence (1:400 dilution Molecular Probes, Eugene, Oregon). Cells were then washed 3 times in PBS and incubated with 1% BSA then incubated overnight in a different primary antibody. The following day they were washed 3 times in PBS and incubated with Alexa 488 chicken anti-rabbit antibody to green fluorescence (1:400 dilution) and washed 3 times in PBS. Slides were allowed to air dry and dehydrated by ethanol and xylene before being coverslipped with Cytoseal (Canada Wide Scientific, Ottawa, ON). Slides were examined with a Zeiss Axioplan 2 at 400x magnification and images captured with Zeiss Axiocam MRc camera and MGrab software. For each colocalization study, regions of 1 mm^2 were randomly counted on 3 slides. Confocal images were collected at 900x magnification on an Olympus FV1000 (Olympus, Canada) and optimized for brightness and contrast in Corel Draw 9 (Corel, Canada).

Drugs

Drugs were applied by a 4-channel gravity feed focal applicator controlled by a Master-8 pulse generator. All chemicals were purchased from Sigma except for the following chemicals. $A\beta_{1-42}$ and reverse peptide $A\beta_{42-1}$, which were purchased from American Peptide Co. (Sunnyvale, USA) and 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP-37157) was purchased from Calbiochem (San Diego, USA). $A\beta_{1-42}$ was prepared from aliquots stored at – 80 °C and used on the day of the experiments. Nicotine was prepared from 100 mM nicotine in ACSF stock solution stored in the dark at room temperature on the day of the experiment. Nicotine was applied via focal applicator. During Ca^{2+} imaging experiments, $A\beta_{1-42}$ was applied both through the focal applicator and in the bath solution.

Statistical Analysis

$[Ca^{2+}]_i$ measurements under control conditions and during drug applications were compared using Student's paired t-test to detect differences between treatments.

RESULTS

Nicotine-Evoked Ca^{2+} Responses in DBB Neurons: Modulation by $A\beta$

The average basal $[Ca^{2+}]_i$ for all cells measured was 99.4 ± 5.5 nM (n = 115). Doses and duration of applications of nicotine were examined to establish an optimal dose that produced a reproducible response of a sufficient amplitude. Figure 2-1A shows a DBB neuron exposed to 10 and 20 mM nicotine for 5 s and Figure 2-1B shows the dose-response relationship between 5 s applications of varying concentrations of nicotine and the corresponding rise in $[Ca^{2+}]_i$. Nicotine has previously been shown to desensitize receptor subtypes, especially the $\alpha 7$ nAChR, however we did not observe desensitization

in our experiments. Control experiments were performed to exclude the contribution of osmolarity-related changes in $[Ca^{2+}]_i$ and to ensure that nicotine applications did not cross-react with muscarinic receptors to alter $[Ca^{2+}]_i$. Neither focal application of iso-osmotic sucrose solutions nor of 500 μM muscarine elicited any change in $[Ca^{2+}]_i$ (Figure 2-2A, B respectively).

Next, the effect of soluble $A\beta_{1-42}$ on the focal application of nicotine was examined. In 16 of 18 cells examined, 100 nM $A\beta_{1-42}$ significantly potentiated nicotinic mediated $[Ca^{2+}]_i$ rises ($p<0.05$; Figure 2-3A). Control nicotinic responses were 63 ± 12 nM, while responses in the presence of 100 nM $A\beta_{1-42}$ increased to 137 ± 25 nM representing a 98 ± 12% increase over controls. The potentiation of nicotinic responses by $A\beta_{1-42}$ was maintained during washout of $A\beta_{1-42}$ and was dose dependent (Figure 2-4). The $A\beta_{1-42}$ potentiation of the nicotine evoked $[Ca^{2+}]_i$ had an EC50 of 10 nM when fitted with a sigmoidal dose-response curve, although the maximum response could be at a higher dose. We observed no significant change in nicotine mediated $[Ca^{2+}]_i$ rises in response to $A\beta_{1-42}$ in 2 of 18 cells (Figure 2-3B). 100 nM $A\beta_{1-42}$ also caused a rise in baseline $[Ca^{2+}]_i$ of 54 ± 14 nM when stimulated with nicotine. Application of 2 concentrations of $A\beta_{1-42}$ alone either via rapid 5 s focal application or by bath application (100 nM, n = 6 and 1 μM n = 5; $p>0.05$) did not change basal $[Ca^{2+}]_i$ (Figure 2-5A, B). In the presence of the reverse peptide, $A\beta_{42-1}$, neither nicotine-evoked $[Ca^{2+}]_i$ nor baseline $[Ca^{2+}]_i$ changed significantly ($p<0.05$; n = 5; data not shown).

Aβ Potentiation of nAChR-Induced Elevation of $[Ca^{2+}]_i$ is Not Mediated by Voltage Gated Ca^{2+} Channels

Previous studies have reported that Aβ may modify Ca^{2+} entry via modulation of Ca^{2+} channels (Silei et al., 1999; Ramsden et al., 2002). Therefore, in the next set of experiments, I bypassed the nAChRs by applying depolarizing concentrations of KCl at doses evoking equivalent rises in $[Ca^{2+}]_i$ as nicotine. A dose-response relationship for KCl-induced elevations in $[Ca^{2+}]_i$ was established and on the basis of this, 1 s applications of 8 mM KCl were used to depolarize the cell. Application of 8 mM KCl yielded an average rise of $[Ca^{2+}]_i$ of 53 ± 10 nM (n = 7) which is similar to that evoked by nicotine (see Figure 2-3). Concomitant application of $A\beta_{1-42}$, with repeated challenges by 8 mM KCl resulted in no significant change in KCl-induced $[Ca^{2+}]_i$ elevation (59 ± 13 nM; n = 5; $p>0.05$; Figure 2-6).

Aβ Potentiates Release of Ca^{2+} from Intracellular Ca^{2+} Stores

The increases of $[Ca^{2+}]_i$ evoked by nicotine may originate from extracellular Ca^{2+} entry via the nAChRs and/or from the release of intracellular Ca^{2+} from stores (Tsuneki et al., 2000; Dajas-Bailador et al., 2002). Thus in the next set of experiments, I examined the source of the nicotine-mediated $[Ca^{2+}]_i$ rise. When cells were perfused with 0 Ca^{2+}/200 μM EGTA, there was a 79.4 ± 5.5% reduction in the nicotinic response ($p<0.05$; n = 7; Figure 2-7A), suggesting that the majority of the nicotine-mediated rise in $[Ca^{2+}]_i$ is mediated by extracellular Ca^{2+} entry. This also demonstrates that there is an intracellular Ca^{2+} component to the nicotine-mediated response. Application of 10 μM of di-(tert-butyl)-1,4-benzohydroquinone (BHQ), a SERCA inhibitor, in perfusing solution containing 0 Ca^{2+}/1 mM EGTA resulted in an initial rise in $[Ca^{2+}]_i$ due to a transient net

efflux of Ca^{2+} from intracellular stores and then completely abolished the nicotine-mediated $[Ca^{2+}]_i$ rise as the intracellular stores are depleted. In fact, at times greater than 1500 s in Figure 2-7B, each application of nicotine caused a small decrease in $[Ca^{2+}]_i$, which may be due to Ca^{2+} efflux via nAChRs due to the reversal of the Ca^{2+} concentration gradient ($p<0.05$; n = 7; Figure 2-7B). In order to test this possibility I performed the same experiment in the presence of non-α7 nAChR antagonist dihydro-beta-erythroidine (5 μM DHβE) and the α7 nAChR antagonist α-bungarotoxin (αBTX). Application of nAChR antagonists did not block the small decrease in $[Ca^{2+}]_i$ observed with nicotine in the presence of BHQ and 0 Ca^{2+}/1 mM EGTA (data not shown). Another possible explanation may be due to the fluorescent properties of nicotine. I observed that focal application of nicotine increased background 340 nm and 380 nm fluorescence, which resulted in an overall decrease in the background ratio. This is unklikely an osmotic effect as previous applications of iso-osmotic did not result in any deflection of the baseline $[Ca^{2+}]_i$.

Previously, nAChR receptors have been shown to be linked to intracellular Ca^{2+} stores via the PLC pathway in SH-SY5Y cells (Dajas-Bailador et al., 2002). In order to determine mechanisms underlying nAChR coupling to intracellular Ca^{2+} stores in DBB neurons, I examined the effect of xestospongin on nAChR responses. Xestospongin has been reported to be an antagonist of IP_3-mediated (inositol 1,4,5-triphosphate) Ca^{2+} release (Reviewed in Verkhratsky, 2005, See Discussion). In the presence of 5 μM xestospongin, nicotine-mediated $[Ca^{2+}]_i$ rises were reduced from 82.9 ± 13.1 nM to 21.8 ± 12.0 nM, which represents an inhibition of 82.3 ± 7.1% (n = 14; $p<0.05$; Figure 2-8A, B). Application of xestospongin also increased the basal $[Ca^{2+}]_i$ from 138.8 ± 13.5 nM to

188.3 ± 19.7 nM, an increase of 38.4 ± 12.7% (n = 14; $p<0.05$). I explored the effect of $A\beta_{1-42}$ on the intracellular Ca^{2+} component of the nicotine-mediated $[Ca^{2+}]_i$ rise further by applying caffeine, an agonist of intracellular Ca^{2+} stores. Focal application of caffeine for 5 s caused an average rise in $[Ca^{2+}]_i$ of 175 ± 17 nM. In the presence of 400 nM thapsigargin (TG), a SERCA pump inhibitor, the caffeine response was abolished ($p<0.05$; n = 5; Figure 2-9A). As with the other SERCA inhibitor BHQ (Figure 2-7B), the application of TG caused a slow and transient elevation in $[Ca^{2+}]_i$ (Figure 2-7A), which can be attributed to transient net efflux from and eventual emptying of intracellular Ca^{2+} stores. In the presence of 100 nM $A\beta_{1-42}$ there was a potentiation of caffeine-mediated $[Ca^{2+}]_i$ rises of 137 ± 37 % and a reversible rise in baseline $[Ca^{2+}]_i$ of 42.0 ± 8.5 nM. In 3 of 5 neurons, no caffeine-mediated $[Ca^{2+}]_i$ rise was observed during washout ($p<0.05$; n = 5; Figure 2-9B).

Application of TG significantly inhibited Aβ mediated potentiation of nAChR mediated $[Ca^{2+}]_i$ rises. In the 5 cells examined, Aβ potentiated the nAChR mediated $[Ca^{2+}]_i$ rises from 37 ± 8 nM to 89 ± 17 nM (141 ± 22%; $p < 0.05$; Figure 2-10A). Application of TG inhibited this response to 29 ± 7 nM, which represents a 68 ± 4 % inhibition. The next set of experiments examined the effect of a mitochondrial release inhibitor CGP-37157 on Aβ potentiation. In 5 cells, with an average potentiation of 97 ± 18%, 25 μM CGP-37157 significantly inhibited this potentiation by 73 ± 7% (n = 5; $p<0.05$; Figure 2-10B). Previously, CGP-37157 was reported to inhibit voltage gated Ca^{2+} channels (Baron and Thayer, 1997). In our experiments, 25 μM CGP-37157 did not inhibit 8 mM K^+ mediated $[Ca^{2+}]_i$ rises. In 4 cells, 8 mM K^+ evoked a 64 ± 18 nM increase, while in the presence of 25 μM CGP-37157 8 mM K^+ evoked a 70 ± 27 nM

$[Ca^{2+}]_i$ rise (p>0.05). Furthermore, CGP-37157 did not inhibit 10 mM caffeine mediated responses (n = 4; p>0.05). In 4 cells, 10 mM caffeine elicited average responses of 117 ± 37 nM, while in the presence of 25 μM CGP-37157 10 mM caffeine response was 111 ± 27 nM.

Pharmacology of Non-α7 and α7 nAChR Receptors on DBB Neurons

I then elucidated the pharmacology of the nicotinic response with the non-α7 nAChR antagonist DHβE and the α7 subunit containing competitive nAChR antagonist αBTX. DHβE (5 μM) inhibited nicotine-mediated rises of $[Ca^{2+}]_i$ in 7 of 12 neurons by 42.8 ± 6.0 (p<0.05 Figure 2-11A). In the presence of 100 nM αBTX, the nicotine response was decreased by 35.8 ± 7.7 % (n = 10), suggesting that nicotine also activates, in part, α7 nAChRs (Figure 2-11B). In the presence of 5μM DHβE and 100 nM αBTX, blocked 70 ± 12 % of the nicotine evoked response (n = 5; Figure 2-12A, B). The residual nicotinic response is similar in amplitude as that observed in the experiment when extracellular Ca^{2+} was removed suggesting that the residual response reflects activation of intracellular Ca^{2+} stores that are not activated by Ca^{2+} influx.

Examination of acutely dissociated cells from the DBB using double-labeling immunohistochemistry revealed the presence of α4 or α7 nAChR subunits on vChAT positive neurons (Figure 2-13A-D). Co-localization of α4 and α7 nAChR subunits on the same DBB neurons was also observed (Figure 2-13 E-G). Cell counts of slides double labeled for α4 and α7 subunits revealed that of the neurons counted expressing at least one type of nAChR subunit, 36.0% were labeled only for the α4 subunit (27/75 neurons), 16.0% were labeled only for the α7 subunit (12/75 neurons) and 48.0% co-expressed both α4 and α7 nAChR subunits (36/75 neurons; Figure 2-13H). Furthermore,

46.5% of ChAT labeled neurons expressed the α4 nAChR subunit and 57.0% of ChAT labeled neurons expressed the α7 nAChR subunit.

DISCUSSION

In this study, focal application of nicotine to acutely dissociated rat basal forebrain neurons increased $[Ca^{2+}]_i$ through a combination of enhanced extracellular Ca^{2+} entry via activation of α4 subunit- and α7-containing nAChRs, and also Ca^{2+} release from intracellular stores with repeated challenges of nicotine. Aβ potentiated the nicotine-mediated $[Ca^{2+}]_i$ rise in the DBB neurons irreversibly, although applications of Aβ alone at concentrations up to 1 μM did not increase $[Ca^{2+}]_i$. Aβ potentiation of nicotine-evoked $[Ca^{2+}]_i$ signals was not due to an enhancement of voltage-dependent Ca^{2+} conductances since Aβ did not potentiate Ca^{2+} entry due to KCl induced depolarization. It appears that the nicotine-evoked rises in $[Ca^{2+}]_i$ involved the release of Ca^{2+} from intracellular stores as such elevations in $[Ca^{2+}]_i$ could be significantly attenuated by both BHQ and xestospongin, a putative IP_3 receptor blocker, which may also inhibit SERCA pumps (Dajas-Bailador et al., 2002; Verkhratsky, 2005). Aβ also potentiated caffeine-mediated rises in $[Ca^{2+}]_i$ suggesting that Aβ increases the release of Ca^{2+} from at least some intracellular stores. Furthermore, Aβ potentiation of caffeine-evoked increases in $[Ca^{2+}]_i$ could be abolished by the SERCA inhibitor TG. TG was also able to block Aβ potentiation of nicotine mediated rises suggesting that Aβ potentiates nicotine and caffeine $[Ca^{2+}]_i$ via overlapping SERCA sensitive stores. Additionally, application of CGP-37157, a mitochondrial Na^+/Ca^{2+} inhibitor also inhibited Aβ potentiation of nicotine mediated rises.

Activation of nAChRs on Basal Forebrain Neurons

nAChRs are present in the basal forebrain and in acutely isolated basal forebrain DBB neurons, and previous studies suggest that nicotinic effects appear to be predominantly mediated via the α4β2 subtype of nAChRs (Fu and Jhamandas, 2003). In the brain slice preparation, application of nicotine increases basal firing rates in GABAergic basal forebrain neurons likely via indirect effects on local circuit glutamate interneurons that express α4β2 nAChRs (Wu et al., 2003). In cells where I studied alterations in $[Ca^{2+}]_i$, the chemical phenotype of the DBB cells (i.e. cholinergic versus GABAergic) was not determined, but a significant number of such neurons showed Aβ potentiation of nicotine-evoked increases in $[Ca^{2+}]_i$. Based on previous data showing the effect of Aβ to be limited to cholinergic DBB neurons, I surmise that the nicotinic effects are likely predominantly on cholinergic cells of the basal forebrain (Jhamandas et al., 2001). Our pharmacological and immunohistochemical experiments demonstrated colocalization of α4 and α7 nAChR subunits in a majority of DBB neurons. Additionally, there was significant colocalization of these subunits with the cholinergic marker ChAT. These data correspond well with a recent study examining electrophysiological responses following activation of these receptors in basal forebrain neurons, which demonstrated the co-localization of both non-α7 and α7 nAChR mediated responses (Henderson et al., 2005). Henderson et al. (2005) also performed immunohistochemistry to demonstrate that the α7 nAChRs were correlated with ChAT positive neurons. Given the high proportion of neurons in the present study that displayed sensitivity to αBTX, this suggests that a significant number of DBB cells that were studied were cholinergic neurons. The doses of nicotine that I used were higher than

other studies. However, the use of acutely dissociated cells (where dendritic processes containing nAChRs may be lost) would require a higher concentration of nicotine to activate a sufficient number of nAChRs on the soma. Additionally, trypsin treatment may decrase the binding affinity of nAChRs on the soma. The high doses were blocked by the nicotinic receptor antagonists. The binding affinity reported for αBTX in competition binding assays for the α7 nAChR is between 0.35-3.5 nM and the binding affinity values reported for DHβE binding to the α4β2 nAChR is between 13.9 and 1900 (Sharples and Wonnacott, 2001). However, in my experiments, these antagonists were capable of inhibiting 10 mM nicotine at 100 nM for αBTX and 10 μM for DHβE. The discrepancy between the binding data and the Ca^{2+} data may be explained the differences in application. In binding studies, the antagonists were applied for hours of incubation and then the nicotine ligand is applied over the course of minutes. However, in my experiments, the antagonists were applied for minutes and the agonists were applied over the course of seconds. These differences in application technique could explain why the observations in my experiements differ from what is expected from the binding affinity of the antagonists.

NAChR Coupling to Intracellular Ca^{2+} Stores

The effect of Aβ on nicotinic receptors has been highly controversial as some studies have shown that Aβ directly activate nAChRs, while others have shown that Aβ inhibits nAChR (Liu et al., 2001; Pettit et al., 2001; Dineley et al., 2002). In addition, whether Aβ affects α7 or non-α7 nAChR subtypes has also been a subject of debate, since initially the effects of Aβ on nAChRs were attributed mostly to α7 nAChRs (Liu et al., 2001), but more recently, non-α7, probably, α4β2 nAChRs, have been shown to be

affected by Aβ (Fu and Jhamandas, 2003; Wu et al., 2004). These studies exclusively examined the effect of Aβ on nAChR currents. In synaptosomes, pretreatment with Aβ inhibited nicotine-mediated Ca^{2+} entry via both non-α7 and α7 nAChRs (Dougherty et al., 2003). In acutely dissociated DBB neurons, both α4 and α7 nAChR subunits have been functionally identified to be present on DBB neurons (Fu and Jhamandas, 2003). This observation was further confirmed in the present study where I identified, using immunohistochemical double labeling, the presence of α4 and α7 subunits on cholinergic DBB neurons and the colocalization of these subunits. The source of $[Ca^{2+}]_i$ rises evoked by nicotine comes from a combination of extracellular activation of both moieties of nAChRs, and also from an interaction between nAChR receptors and intracellular Ca^{2+} stores. The effect of 100 nM $A\beta_{1-42}$ in potentiating nicotine-induced responses is specific to nAChRs as no potentiation was observed with KCl-mediated rises in $[Ca^{2+}]_i$. Activation of VGCC by KCl in DBB has previously been shown not to activate any TG-sensitive stores as simultaneous voltage clamp and Ca^{2+} imaging experiments have demonstrated that $[Ca^{2+}]_i$ rises are not changed in the presence of TG (Murchison and Griffith, 1998).

Aβ and $[Ca^{2+}]_i$ Homeostasis

Previous studies have shown that Aβ alters Ca^{2+} homeostasis (Mattson et al., 1992). Mattson et al. (1992) reported that Aβ fragments enhance glutamate-mediated $[Ca^{2+}]_i$ rise and excitotoxic neuronal death, however, sole application of Aβ fragments did not raise $[Ca^{2+}]_i$ entry. On the other hand, Brorson et al. (1994) also showed that Aβ fragments enhances glutamate-mediated Ca^{2+} and Aβ fragments evoked $[Ca^{2+}]_i$ rises. In our preparation, focal applications of Aβ alone at concentrations up to 1 μM did not alter

$[Ca^{2+}]_i$ as some other studies have reported (Brorson et al., 1995; Kawahara et al., 2000). However, neither of the aforementioned studies examined actions of the isoform $A\beta_{1-42}$, which is the more pathophysiologically relevant of the Aβ species. Moreover, the study by Kawahara et al. (2000) utilized an immortal hypothalamic cell line where Aβ formed *de novo* Ca^{2+}-permeable pores, a finding not observed in preparations other than planar lipid bilayers (Arispe et al., 1996). Recently, application of $A\beta_{1-42}$ for up to six hours was shown not alter $[Ca^{2+}]_i$ in cultured hippocampal neurons (Abramov et al., 2003).

Although I did not observe that application of Aβ-induced Ca^{2+} rises, I did observe that Aβ was able to potentiate nAChR and caffeine-evoked increases in $[Ca^{2+}]_i$ which involves the repeated release of Ca^{2+} from intracellular stores. Moreover, the potentiation with repeated nicotine applications became larger as the baseline $[Ca^{2+}]_i$ rose gradually during and after $A\beta_{1-42}$ application. $A\beta_{1-42}$ application also potentiated repeat activation of intracellular Ca^{2+} stores by caffeine, however, during washout of $A\beta_{1-42}$, caffeine no longer mediated $[Ca^{2+}]_i$ rises. Aβ potentiation of both caffeine and nicotine-induced $[Ca^{2+}]_i$ rises could be blocked by thapsigargin. Furthermore, Aβ potentiation of nicotine responses could be blocked by the mitochondrial Na^+/Ca^{2+} inhibitor CGP-37157.

The phenomena that nicotinic responses are potentiated irreversibly by Aβ whereas caffeine responses are transiently potentiated may be related to differences in the total load of cytoplasmic Ca^{2+} and saturation of Ca^{2+} buffers. Repeated application of nicotine results in a significant entry of extracellular Ca^{2+}, which would result in an increase in baseline $[Ca^{2+}]_i$ and transient $[Ca^{2+}]_i$ elevations from repeated activation of nAChRs. Additionally, each activation of nAChRs by nicotine also results in the release of Ca^{2+} from intracellular stores (Dajas-Bailador et al., 2002). When intracellular stores

of $[Ca^{2+}]_i$ are repeatedly released and eventually depleted, a mechanism whereby Aβ can potentiate nicotine-evoked $[Ca^{2+}]_i$ rises could result from progressive overfilling of the nicotine-sensitive stores. Our study shows that Aβ is mediating the overfilling of TG-sensitive stores by increasing Ca^{2+} release from the Na^+/Ca^{2+} exchanger in mitochondria located in close proximity to TG-sensitive stores. The exact mechanism of Aβ action is not known, however, I propose that Aβ could be acting upon mitochondria to cause overfilling resulting in the excess release of Ca^{2+} via the Na^+/Ca^{2+} exchanger, resulting in overfilling of TG sensitive stores. Saturation of the buffers may also play a role in maintaining the elevation. Previous studies have demonstrated that ER-mitochondrial interactions are important for continued filling of the ER (Arnaudeau et al., 2001; Csordas and Hajnoczky, 2001; Smaili et al., 2001).

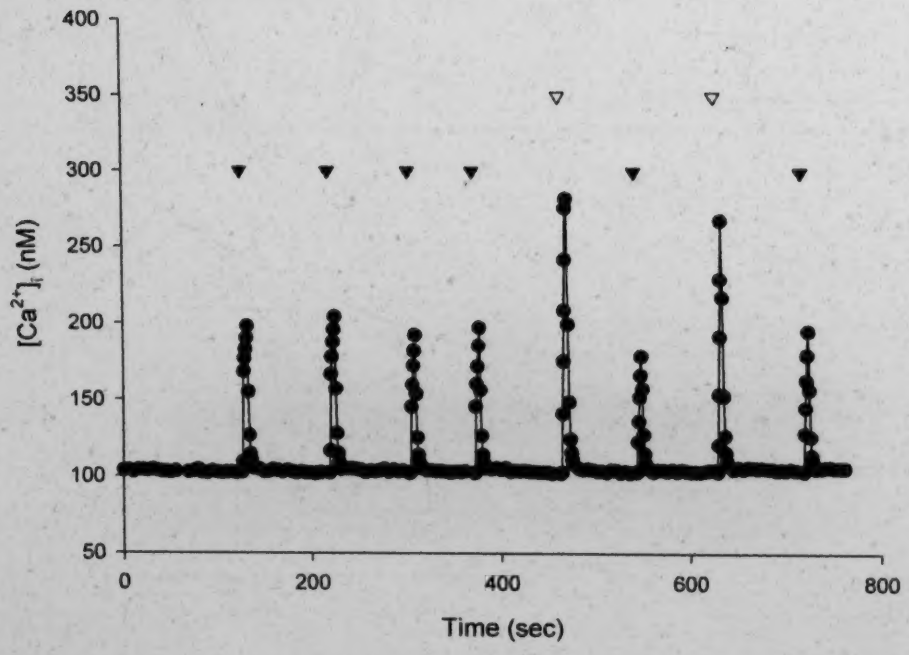
Repeated application of caffeine activates release of Ca^{2+} from caffeine-sensitive stores via ryanodine receptors. In the absence of any additional entry of extracellular Ca^{2+}, the overall result of repeatedly activating Ca^{2+} stores would be the gradual overall loss of cytoplasmic Ca^{2+} via plasma membrane pumps or exchangers. The transient enhancement of the caffeine response by Aβ is consistent with the idea that there is only a transient overfilling of caffeine-sensitive stores and no net increase in the total cytoplasmic Ca^{2+} load (which also suggested that there was no persistent activation of a Ca^{2+} release activated -like channel).

Functional Implications

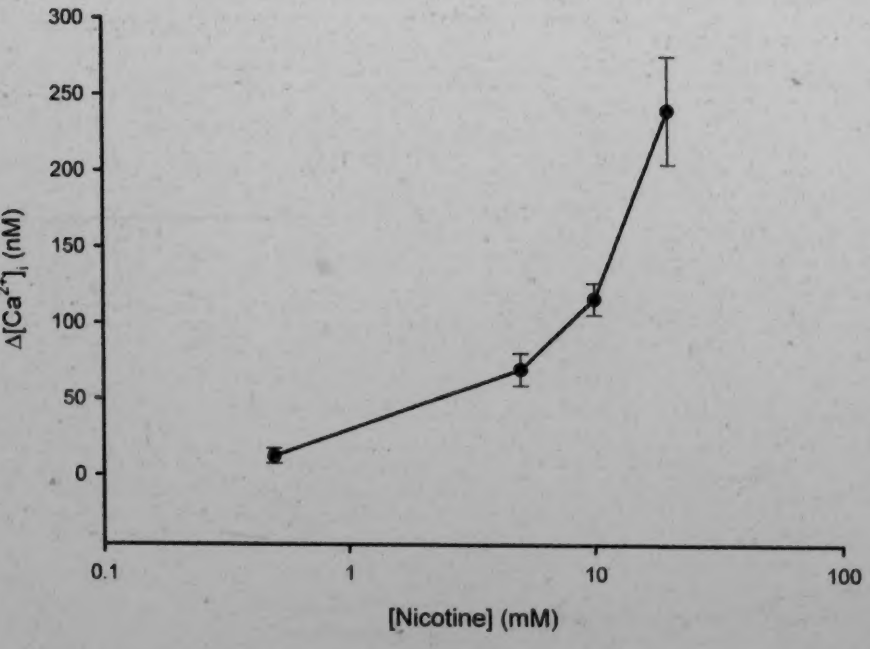
In our study, I showed that Aβ potentiates nicotine-evoked $[Ca^{2+}]_i$ rises via overfilling of mitochondria. This effect is only prominent with repeated activation of intracellular Ca^{2+} stores and on a large cytoplasmic Ca^{2+} load via extracellular Ca^{2+} entry.

Given the tight regulation of Ca^{2+} signaling in neurons, the increase in $[Ca^{2+}]_i$ triggered by nAChR activation may cause dysfunction in the basal forebrain neurons prior to deposition, but not necessarily in the absence of amyloid plaques due to the presence of soluble forms of Aβ. There is general agreement that the neurotoxic effects of Aβ may, in part, occur via its dysregulation of intracellular Ca^{2+} homeostasis, thus Aβ-induced enhancement of $[Ca^{2+}]_i$ via nAChRs on basal forebrain neurons may render them vulnerable to neurodegeneration in AD. Further studies examining the role of overloading endoplasmic reticulum Ca^{2+} stores and its role in neurotoxicity in the basal forebrain may lead to treatments to protect against the degeneration of basal forebrain in AD.

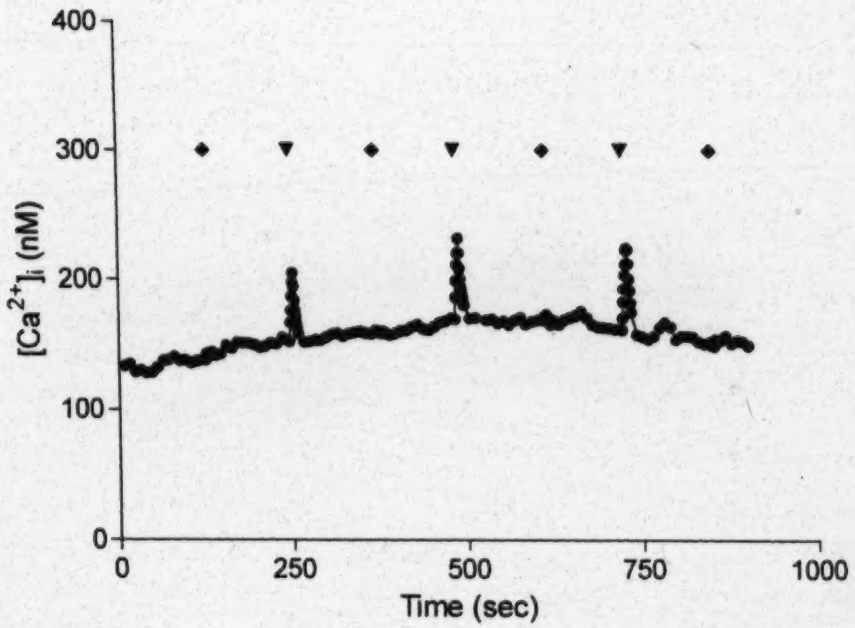
A.


400
350
300
250
200
150
100
50
$[Ca^{2+}]_i$ (nM)
0
200
400
600
800
Time (sec)

B.


300
250
200
150
100
50
0
$\Delta[Ca^{2+}]_i$ (nM)
0.1
1
10
100
[Nicotine] (mM)

A.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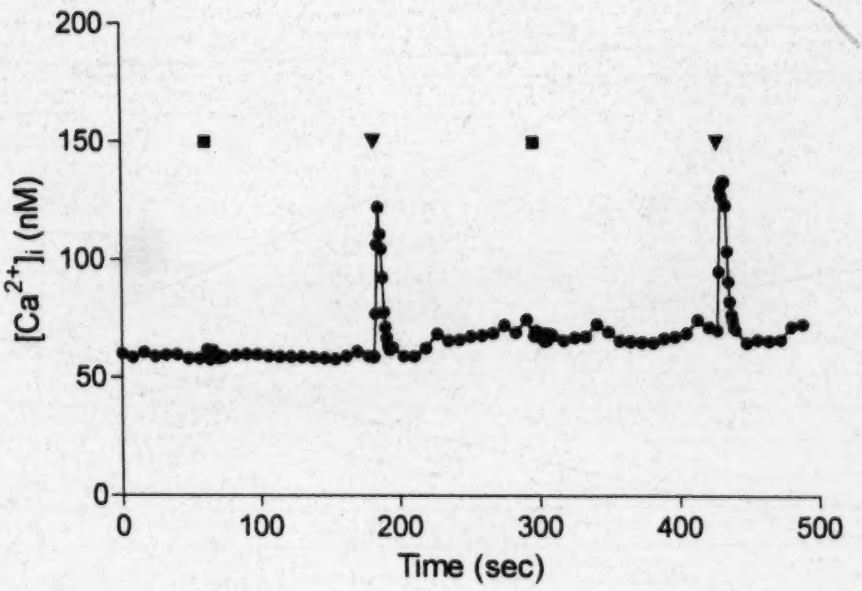
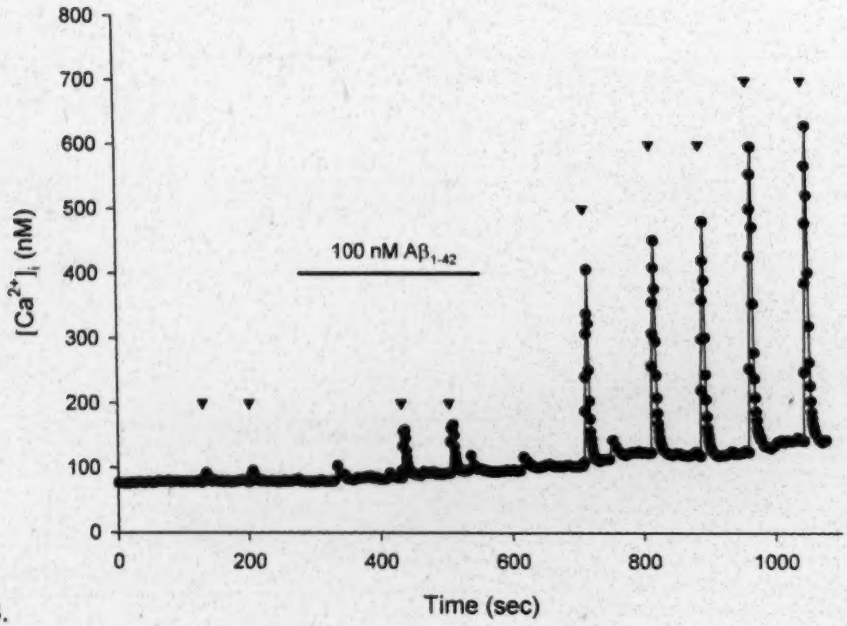
300
200
100
0
$[Ca^{2+}]_i$ (nM)
0
250
500
750
1000
Time (sec)

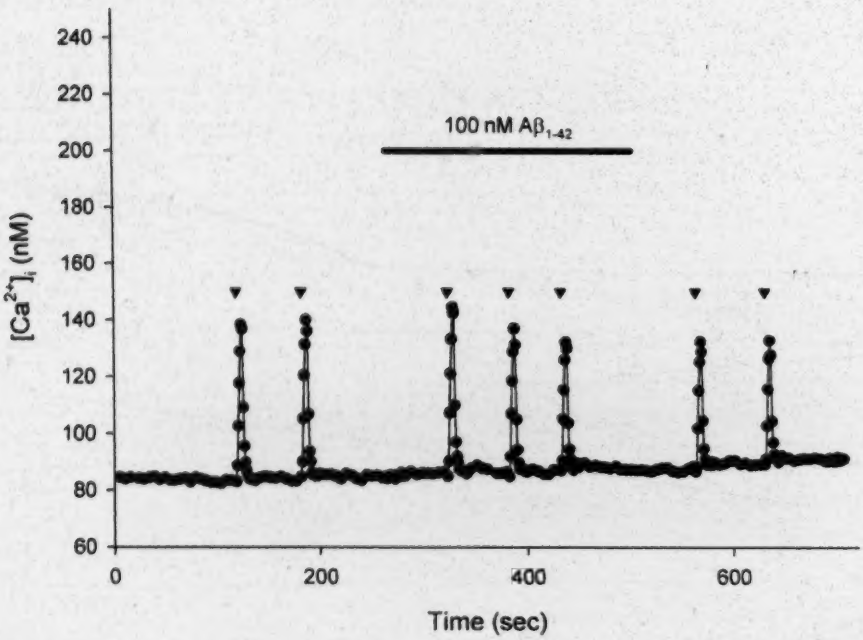
B.

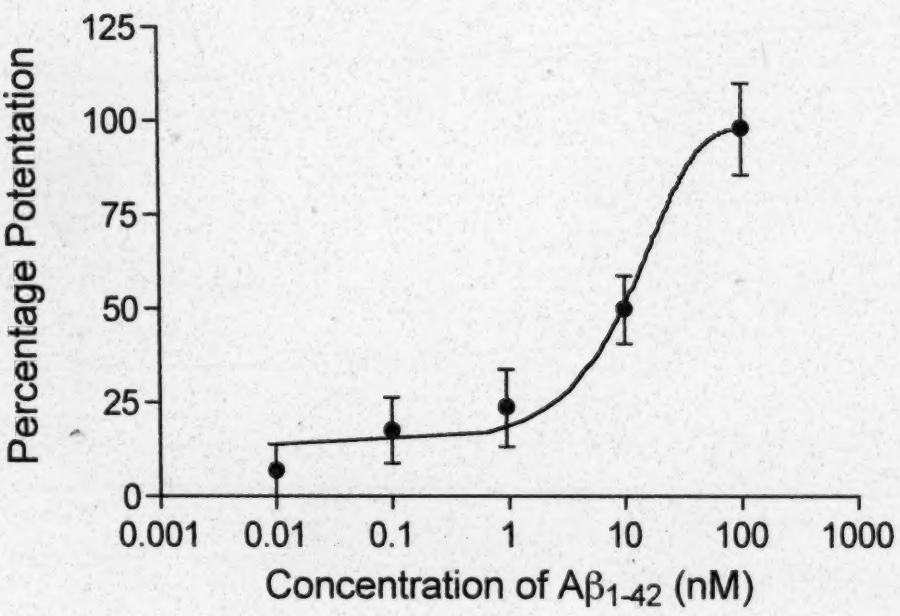

200
150
100
50
0
$[Ca^{2+}]_i$ (nM)
0
100
200
300
400
500
Time (sec)

A.

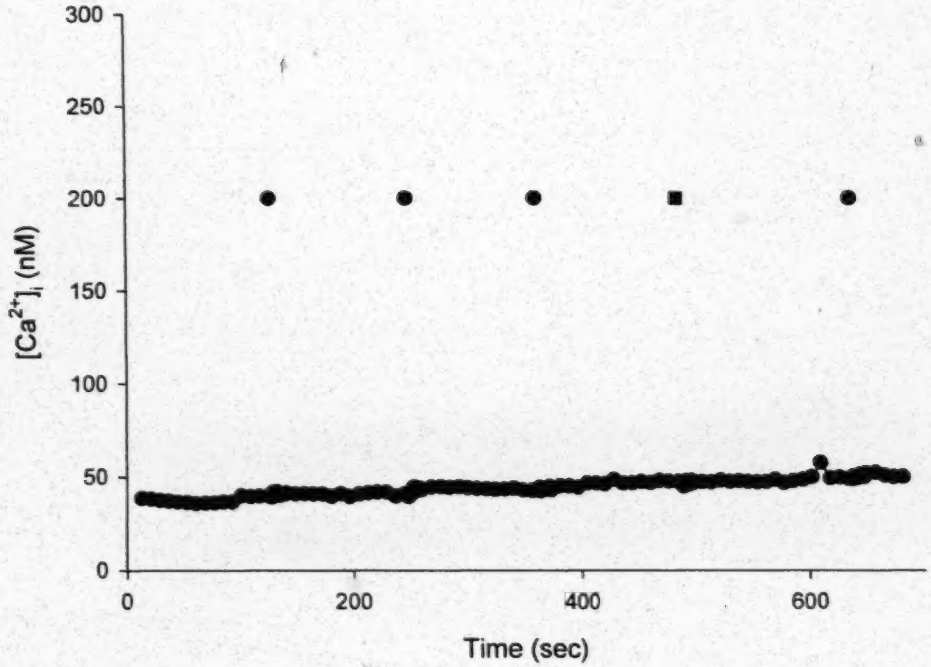

800
700
600
500
400
300
200
100
0
$[Ca^{2+}]_i$ (nM)
100 nM $A\beta_{1-42}$
0
200
400
600
800
1000
Time (sec)

B.

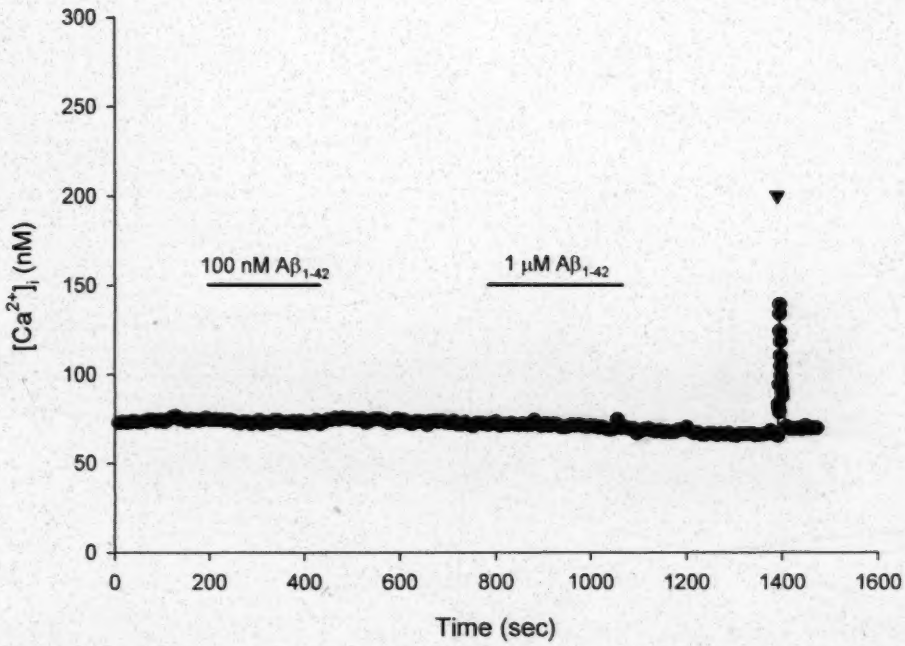

240
220
200
180
160
140
120
100
80
60
$[Ca^{2+}]_i$ (nM)
100 nM $A\beta_{1-42}$
0
200
400
600
Time (sec)

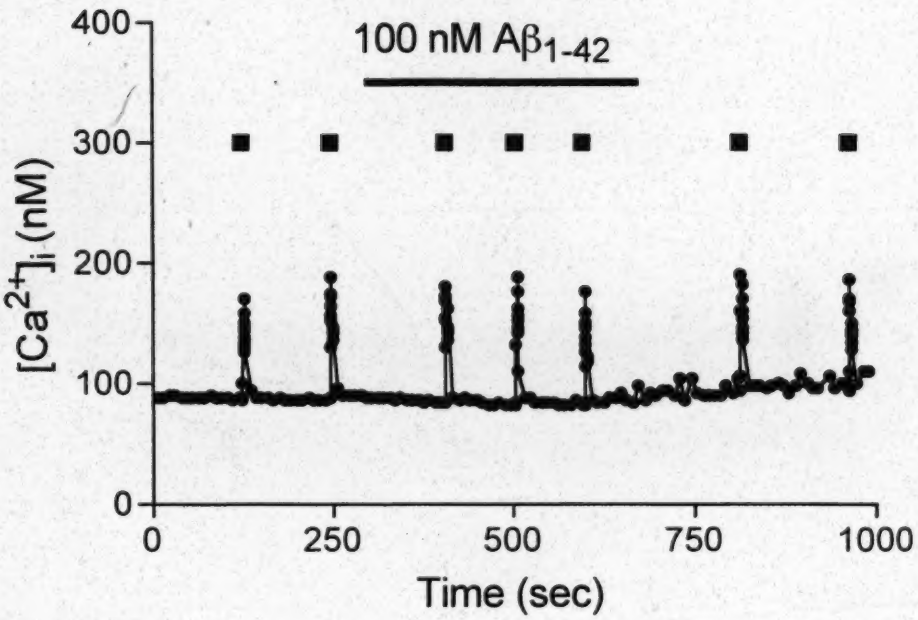

Percentage Potentation
125
100
75
50
25
0
0.001
0.01
0.1
1
10
100
1000
Concentration of Aβ1-42 (nM)

A.

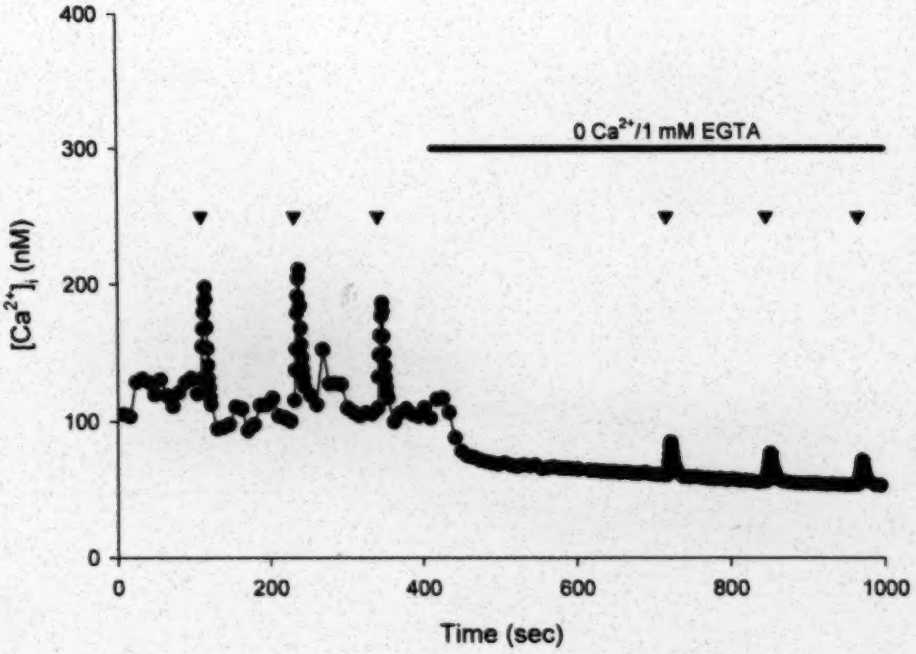

300
250
200
150
100
50
0
$[Ca^{2+}]_i$ (nM)
0
200
400
600
Time (sec)

B.

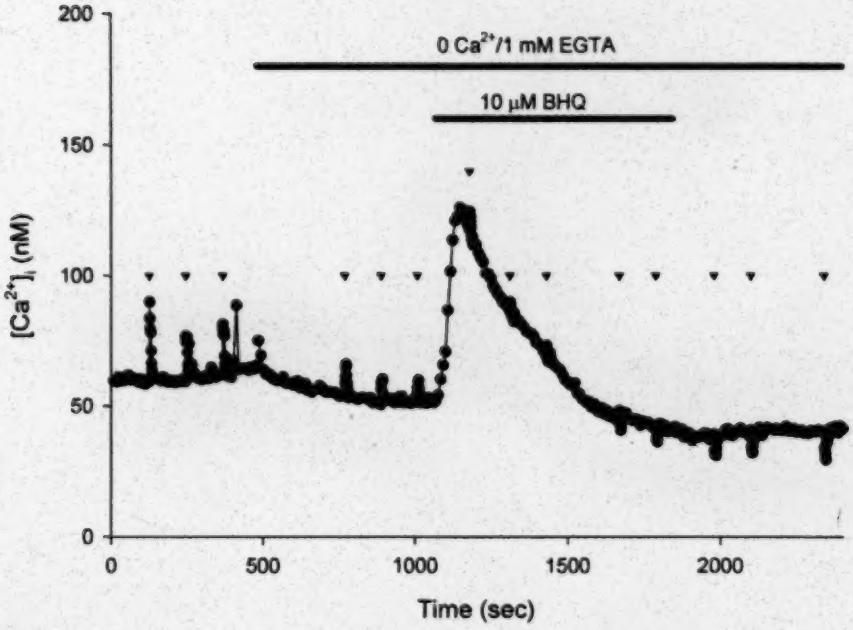

300
250
200
150
100
50
0
$[Ca^{2+}]_i$ (nM)
100 nM $A\beta_{1-42}$
1 μM $A\beta_{1-42}$
0
200
400
600
800
1000
1200
1400
1600
Time (sec)


100 nM Aβ1-42
400
300
200
100
0
[Ca2+]i (nM)
0
250
500
750
1000
Time (sec)

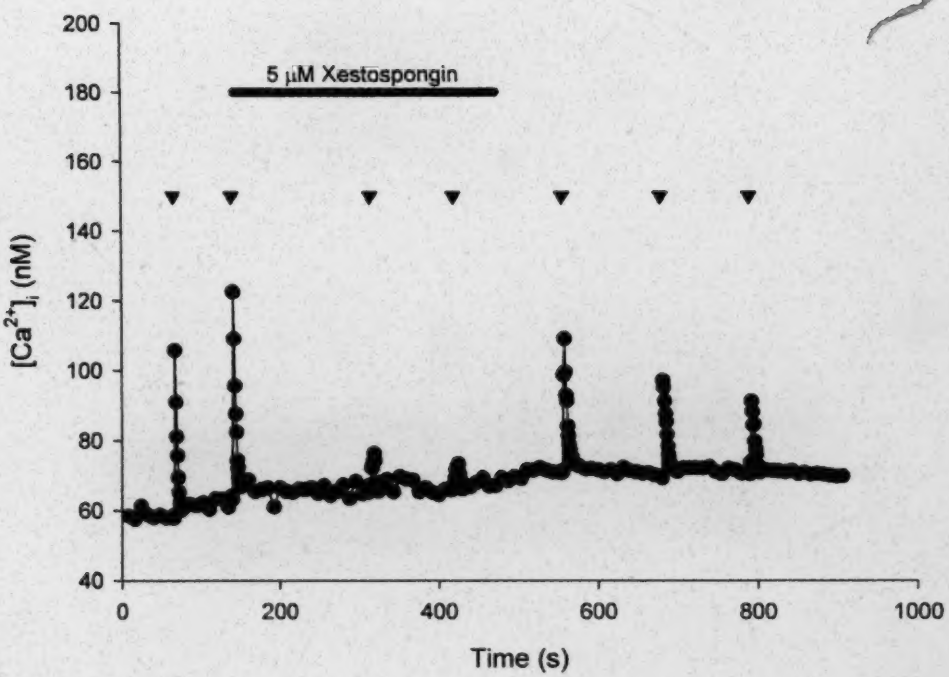
A.


400
300
200
100
0
$[Ca^{2+}]_i$ (nM)
0 Ca^{2+}/1 mM EGTA
0
200
400
600
800
1000
Time (sec)

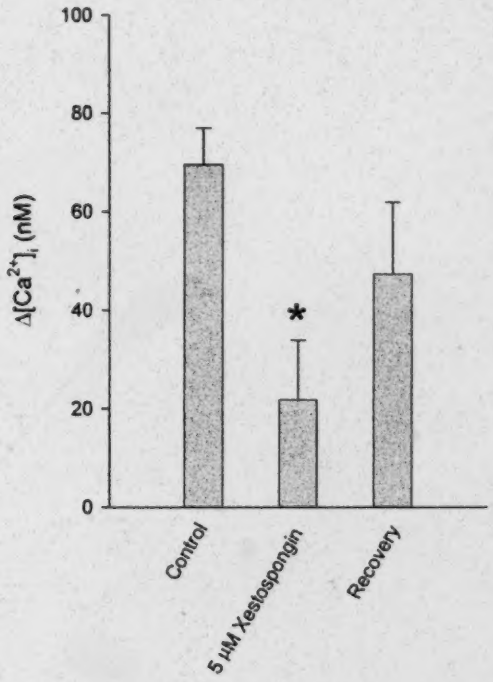
B.


200
150
100
50
0
$[Ca^{2+}]_i$ (nM)
0 Ca^{2+}/1 mM EGTA
10 μM BHQ
0
500
1000
1500
2000
Time (sec)

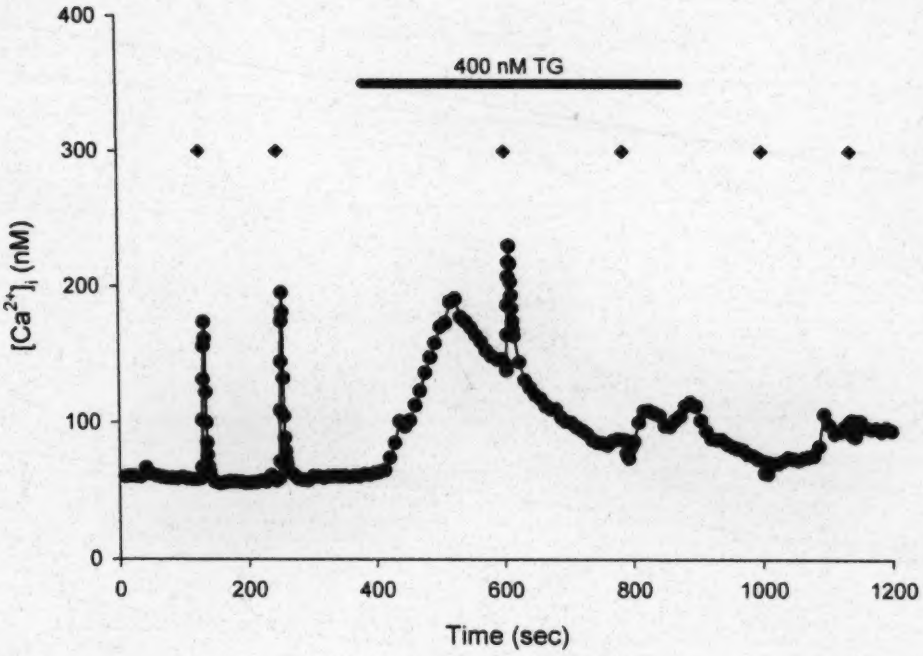
A.


5 μM Xestospongin
200
180
160
140
120
100
80
60
40
$[Ca^{2+}]_i$ (nM)
0
200
400
600
800
1000
Time (s)

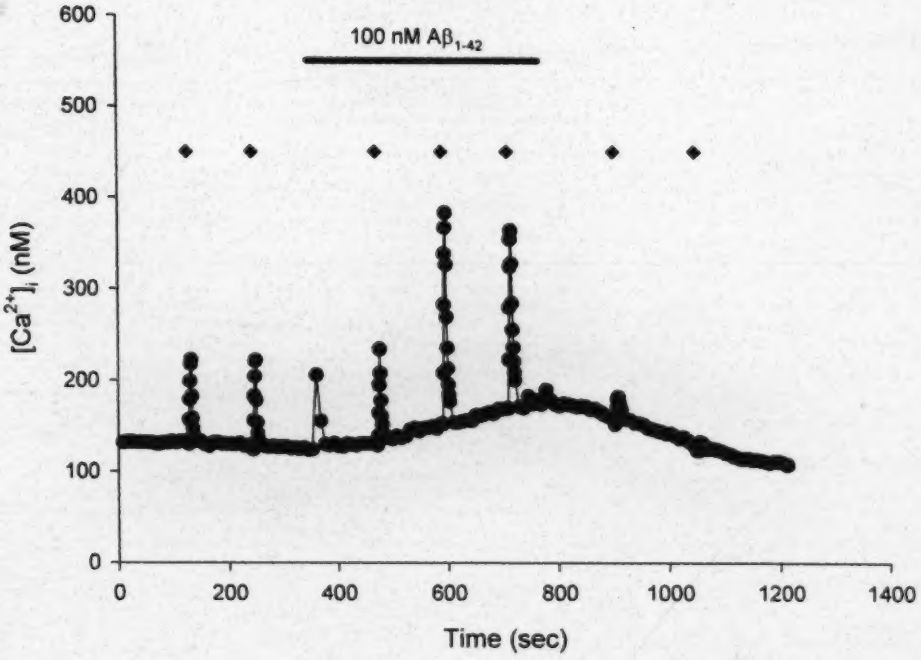
B.


100
80
60
40
20
0
$\Delta[Ca^{2+}]_i$ (nM)
*
Control
5 μM Xestospongin
Recovery

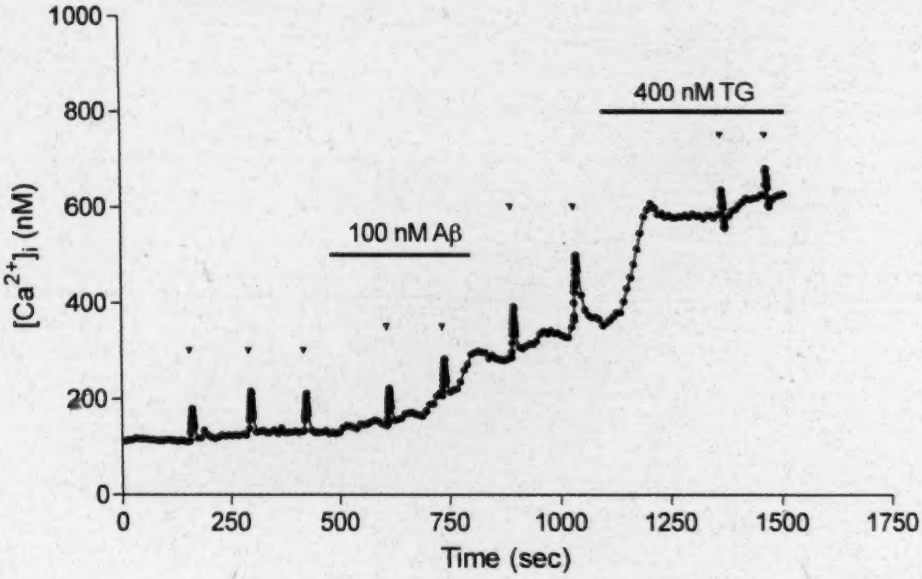
A.


400 nM TG
400
300
200
100
0
$[Ca^{2+}]_i$ (nM)
0
200
400
600
800
1000
1200
Time (sec)

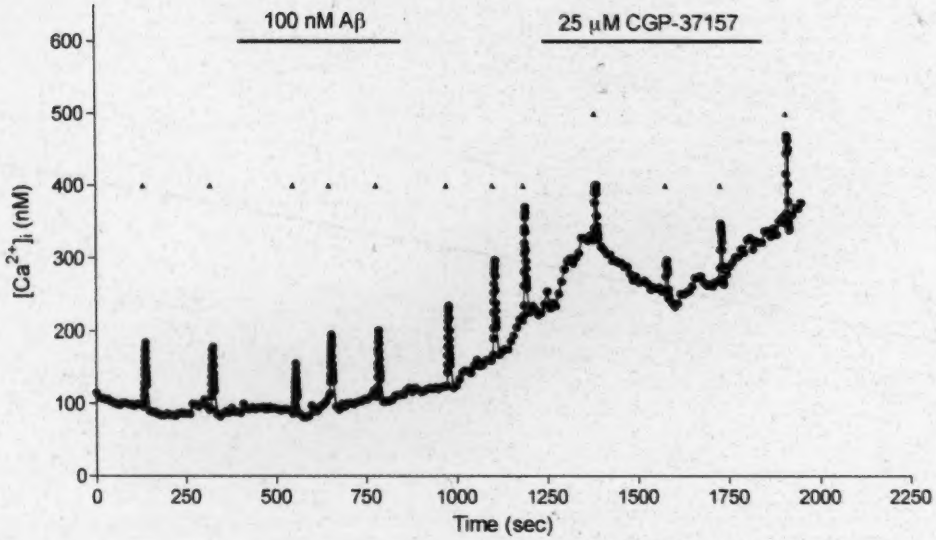
B.


100 nM $A\beta_{1-42}$
600
500
400
300
200
100
0
$[Ca^{2+}]_i$ (nM)
0
200
400
600
800
1000
1200
1400
Time (sec)

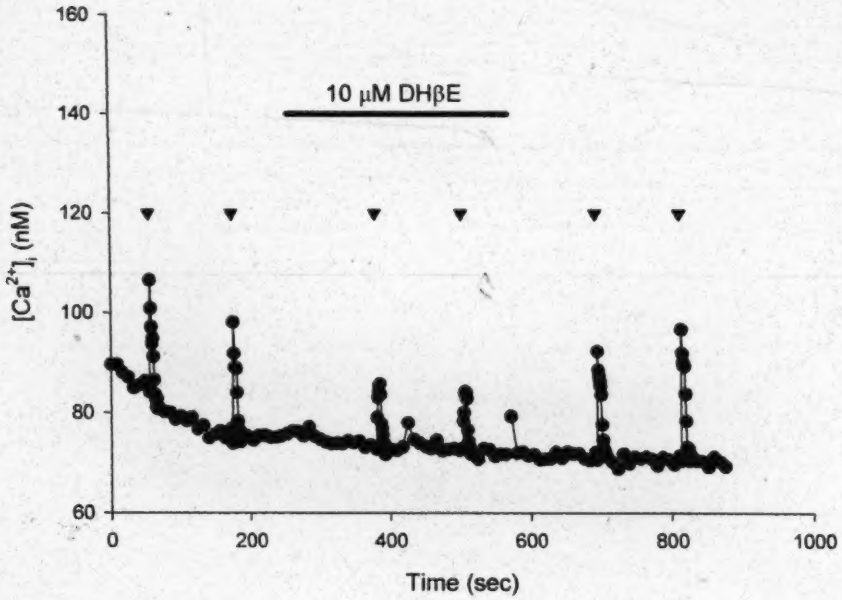
A.


1000
800
600
400
200
0
$[Ca^{2+}]_i$ (nM)
400 nM TG
100 nM Aβ
0
250
500
750
1000
1250
1500
1750
Time (sec)

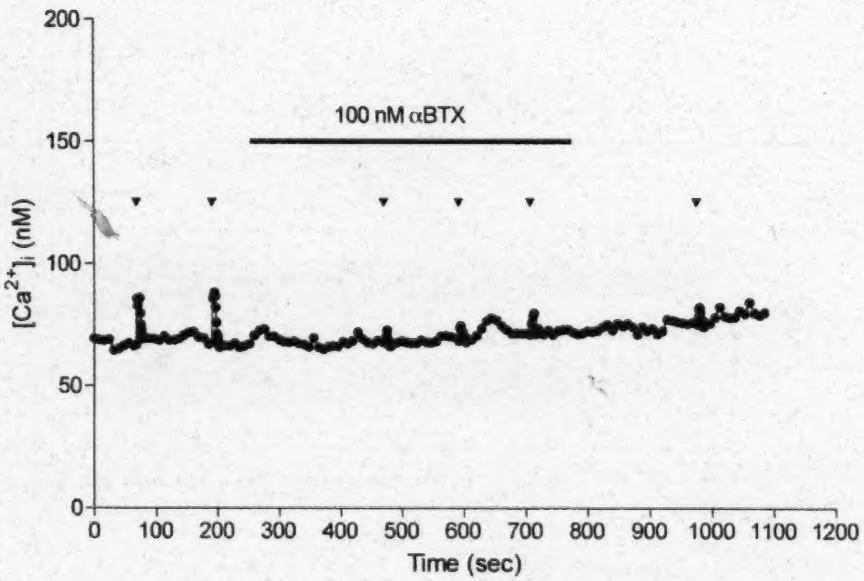
B.


600
500
400
300
200
100
0
$[Ca^{2+}]_i$ (nM)
100 nM Aβ
25 μM CGP-37157
0
250
500
750
1000
1250
1500
1750
2000
2250
Time (sec)

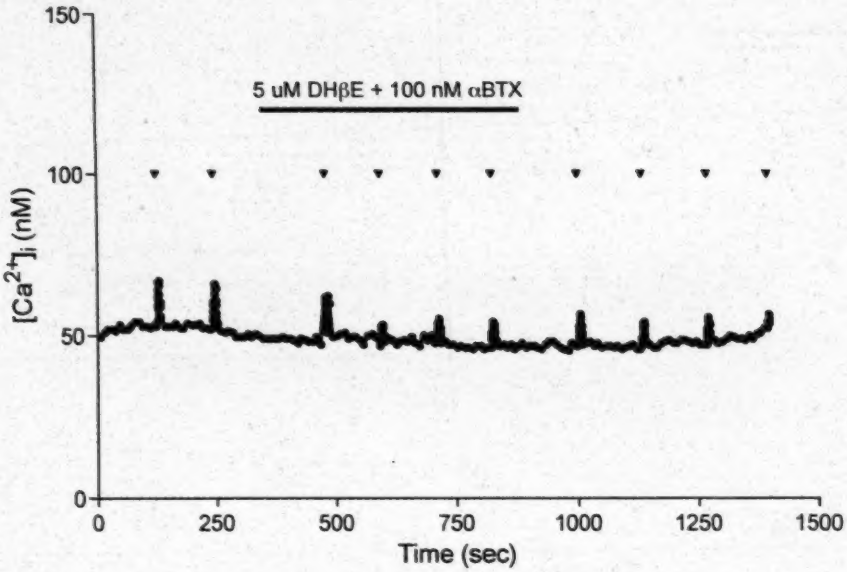
A.


10 μM DHβE
[Ca2+]i (nM)
160
140
120
100
80
60
0
200
400
600
800
1000
Time (sec)

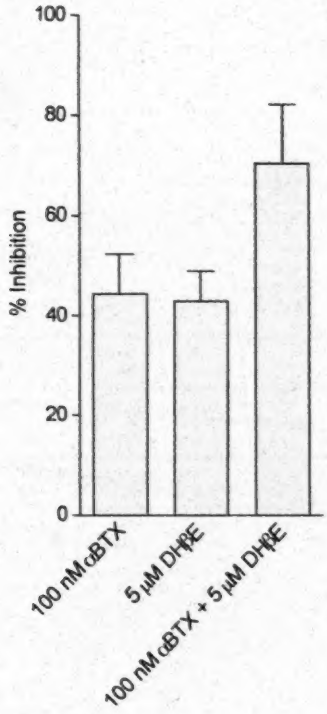
B.


100 nM αBTX
[Ca2+]i (nM)
200
150
100
50
0
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
Time (sec)

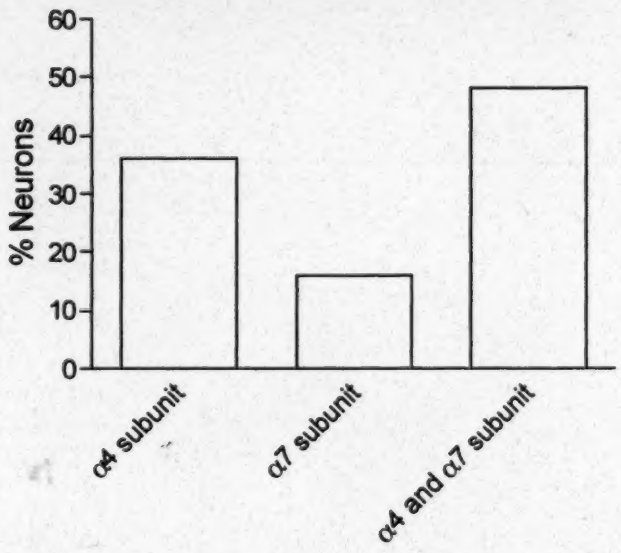
A.


150
100
50
0
5 uM DHβE + 100 nM αBTX
[Ca2+]i (nM)
0
250
500
750
1000
1250
1500
Time (sec)

B.


100
80
60
40
20
0
% Inhibition
100 nM αBTX
5 μM DHβE
100 nM αBTX + 5 μM DHβE

H.


60
50
40
30
20
10
0
% Neurons
α4 subunit
α7 subunit
α4 and α7 subunit

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Chapter 3

Synaptic Effects of β-Amyloid on Glutamate Neurotransmission in the Rat Cholinergic Basal Forebrain

INTRODUCTION

Alzheimer's disease (AD) is disease characterized by a progressive decline in memory and other cognitive functions such as language and perception in elderly individuals (McKhann et al., 1984). β-amyloid peptide (Aβ), a 39-43 amino acid peptide, is a critical pathological mediator of the synaptic dysfunction, synaptic loss and neuronal death observed in AD (Small et al., 2001; Selkoe, 2002). The pathology of AD starts with mild synaptic dysfunction and eventually progresses to neuronal death, however no receptor has yet been identified to mediate the effects of Aβ on synaptic activity (Small et al., 2001). A potential candidate is the nicotinic acetylcholine receptor (nAChR) as studies have suggested that Aβ binds to this receptor and mediates electrophysiological effects (Wang et al., 2000a; Dineley et al., 2001; Pettit et al., 2001; Fu and Jhamandas, 2003)

Several lines of evidence suggest that the cognitive impairment observed early on in AD is mediated by Aβ induced synaptic dysfunction. Pathological examination of AD brains has revealed that specific neurotransmitter systems are targeted in AD. The most well studied system is the cholinergic basal forebrain neurons, which are especially prone to neurodegeneration (Whitehouse et al., 1982). Additionally, examination of brains from AD individuals 2-4 years after diagnosis reveal significant reductions of synaptic density in the temporal and frontal lobe (Davies et al., 1987; Hamos et al., 1989; Masliah et al., 1991). In individuals with mild cognitive impairment or very mild AD, there was a significant reduction in synaptic density when compared with control individuals (Masliah et al., 2001). Furthermore, studies examining the cognitive decline in AD and neuropathology reveal that the reduction of synaptic density is more closely correlated

with the decline in cognitive function of AD individuals than the deposition of Aβ plaques (Terry et al., 1991). Studies examining the level of insoluble Aβ demonstrated either a weak correlation or no correlation between Aβ plaque load and cognitive decline (Katzman et al., 1988; Morris et al., 1991; Terry et al., 1991). However, a correlation between Aβ and the degree of cognitive decline was found when both the insoluble and soluble forms of Aβ were measured (Lue et al., 1999; Naslund et al., 2000). These studies demonstrate that both the accumulation of Aβ and synaptic loss occur early on AD and may be responsible for the initial mild cognitive decline observed.

Deficits in basal synaptic transmission and long term potentiation (LTP), a form a synaptic plasticity, prior to the development of Aβ plaques have also been observed in transgenic mice that overexpress Aβ. These mice express mutant forms of the human amyloid precursor protein that result in overexpression of Aβ. In the V717F transgenic model, there is a significant reduction in synaptic excitatory postsynaptic potentials and rapid decay of LTP compared with control animals at 4-5 months of age prior to the formation of Aβ plaques, suggesting that soluble Aβ isoforms mediate this action (Chen et al., 2000). Although such transgenic mice do not display all the features of AD pathology such as neurofibrillary tangles consisting of hyperphosphorylated tau protein, the synaptic loss observed early on in these animals fits well with the pathological observations made in brains from mildly cognitive impaired individuals. Furthermore, *in vitro* experiments have shown that soluble oligomers of Aβ are capable of inhibiting LTP (Walsh et al., 2002). Taken together, these data demonstrate the importance of soluble forms of Aβ in mediating synaptic dysfunction. However, there is at present little information on how Aβ may influence normal synaptic transmission in the brain,

particularly in structures such as the cholinergic basal forebrain, that are at the epicenter of the chemical pathology seen in AD.

Currently, no receptor has been definitively identified to mediate Aβ actions on synaptic function, although many have been proposed. One possible candidate receptor is the nicotinic acetylcholine receptor (nAChR), which has been demonstrated to be involved in neuromodulation (McGehee et al., 1995). There are very few examples of primary nicotinic mediated EPSC in the brain and these include hippocampal interneurons, the medial vestibular nucleus and the nucleus ambiguous (Phelan and Gallagher, 1992; Zhang et al., 1993; Frazier et al., 1998). In this project, I examined the DBB for nAChR mediated EPSCs, but did not find any and thus I focused on nicotinic modulation of glutamate EPSCs. Aβ has been shown to exhibit significant affinity to these receptors and electrophysiological experiments have shown that Aβ is capable of influencing the function of these receptors (Pettit et al., 2001; Fu and Jhamandas, 2003). I will examine the effect of $A\beta_{1-42}$ on synaptic transmission in cholinergic neurons of the basal forebrain identified with the fluorescent dye Cy3 192IgG or with electrophysiological criteria. The aims of this project are 1) to examine the effect of soluble $A\beta_{1-42}$ on spontaneous miniature post-synaptic excitatory events (mEPSCs) in cholinergic basal forebrain neurons and 2) to determine if Aβ effects on spontaneous excitatory events are mediated by nAChRs, and if so, what specific subtypes of nAChRs are involved.

MATERIALS AND METHODS

Cy3-192 IgG Neuron Labeling

The majority of neurons used in this study (43/50) were identified with Cy3-192 IgG (Advanced Targeting Systems, San Diego, USA) which is an inert fluorescent dye conjugated to an antibody that binds to the p75 neurotrophin receptor expressed only in cholinergic neurons of the basal forebrain. Following intracerebroventricular injection, Cy3-192 IgG retrogradely labels only cholinergic neurons of the basal forebrain that project to the hippocampus (Hartig et al., 1998a; Wu et al., 2000). Injection of Cy3-192 IgG was performed based upon a previously described protocol (Wu et al., 2000). 22-27 day post-natal Sprague-Dawley rats (50-70 g) were anaesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg; 0.05% Somnotol, MTC Pharmaceuticals, Hamilton, Canada) and then injected subcutaneously with 0.02% of atropine. The rats were then placed in a stereotaxic frame (Narishige, Tokyo, Japan) and 5 µl of 1:1 diluted Cy3-192 IgG was injected into the left and right ventricles (1.1 mm posterior to Bregma, 1.2 mm lateral from the midline, and 2.6-3.7 mm below the dura). All procedures were approved by University of Alberta Health Sciences Laboratory Animal Services (Protocol number 154/04/05).

Diagonal Band of Broca Slice Preparation

Brain tissue was removed from Sprague-Dawley rats previously injected with Cy3-192 IgG between three and seven days after injections based on previously described procedures (Easaw et al., 1997). Briefly, animals were anaesthetized with halothane and decapitated. The brain was quickly removed and placed in a 3-5 °C bicarbonate buffered solution that contained (in mM): 140 NaCl, 2.5 KCl, 12 $MgCl_2$, 1.2 NaH_2PO_4 and 2.4

$CaCl_2$, 25 mM HCO_3 and 11 mM D-glucose (pH 7.4). All solutions were oxygenated by bubbling with a mixture of 95% O_2 and 5% CO_2. 300 μM thick longitudinal brain slices were cut with a vibratome (Slicer HR2, Sigmann Elektronik, Germany) and then incubated for one hour at 32 °C in artificial cerebrospinal fluid (ACSF) prior to recording (in mM; NaCl 126, 2.5 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 25 $NaHCO_3$ and 11 mM D-glucose; pH 7.4).

Recordings from DBB Slices

DBB slices were bath perfused with ACSF (23-25°C) at a rate of 1 ml/min and visualized under an Axioscope 2 Fs microscope (Zeiss, Germany) at 60x magnification. Cy3-192 IgG labeled neurons were selected using the appropriate filter for Cy3 (546 nm excitation and 575-640 nm emission). Individual neurons were then visualized under 60x magnification using differential infrared contrast optics. Cells were recorded using whole cell patch clamp technique. The internal pipette solution was composed of the following (in mM): 140 K^+-gluconate, 2 KCl, 5 HEPES, 5 MgATP, 0.5 NaGTP, 10 EGTA and pH was raised to between 7.2 and 7.3 with potassium hydroxide. The internal solution had an osmolarity of 280 mM. Patch clamp electrodes (World Precision Instruments, Sarasota, USA – Thin Wall with Filament, 1.5 mm) were pulled with a Narishige (PP-83) puller to yield electrodes with resistances of 4-8 MΩ. Recordings were made from the DBB, where seals of 1 GΩ or greater were obtained using a Siskiyou Design Instruments 4 axis motorized micromanipulator (MX 831; Grants Pass, Oregon). Using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale CA), Cy3-192IgG labeled cells were initially held in voltage-clamp mode at -60 mV. Recordings were made at a bandwidth of 10 kHz and filtered with a 2 kHz low pass Bessel filter.

Prior to performing voltage-clamp recordings, in 7 of 50 neurons, neurons were held in current clamp and 2 second current injections of 0.2 nA steps from -0.6 nA to + 0.6 nA were performed in the absence of tetrodotoxin (TTX; Figure-3-1A). The average resting membrane potential for these cells was -53.9 ± 5.1 mV (n = 7). Cholinergic neurons were identified based upon previously described criteria such as lack of a hyperpolarizing sag and the presence of burst firing upon depolarization (Wu et al., 2000). A gap free protocol was used to hold cells at either -60 or -80 mV and recordings were performed in the presence of 1 μM TTX and either 10 μM of bicuculline or 50 μM picrotoxin, $GABA_A$ receptor antagonists. Baseline recordings for 5 minutes were observed to ensure stability of the cell. Each recorded treatment was between 5 and 7 minutes, with a 2 minute interval between treatments so that at least 100 events were recorded. Seal and voltage ramp protocols were run between treatments. Seal tests were performed between treatments to monitor cell access was maintained. Voltage ramps consisted of hyperpolarizing the cells to -110 mV, and then depolarizing them to +30 mV over the course of 8 seconds (Figure 3-1B).

Statistical Analysis

Miniature excitatory post-synaptic currents were recorded using pClamp 9.0 and analyzed with Clampfit 9.2 using a template search for events (Clements and Bekkers, 1997). A template was created based upon the average of 10 recorded events. The template search parameter was set between 4.0 and 5.0 and events were then inspected visually for removal of extraneous events. Student's paired and unpaired t-test was used to compare treatments and parameters between cell populations respectively. The significance level for the t-tests was set at $p<0.05$. Kolmogorov-Smirnov (KS) testing

was also used to examine differences and the significance level of this test was set at $p<0.0005$. The mEPSC parameters illustrated in Figure 3-1 were measured. Peak amplitude of the mEPSC was measured (Figure 3-1(a)). The rise time from 10% of the peak amplitude to 90% of the peak amplitude was measured (Figure 3-1(b)) and the decay time from 90% of the peak amplitude to 10% of the peak amplitude was measured (Figure 3-1(c)) and the area between the baseline and the event was also measured (Figure 3-1(d)). The conductance of was calculated from voltage ramps by taking perfomring linear regression of the current-voltage relationship between -110 mV and -60 mV.

Immunohistochemistry

Rats previously injected with Cy3-192 IgG were anaesthetized with halothane and intraperitoneal urethane was administered. Animals were then exsanguinated with phosphate buffered saline (PBS) and infused with 4% formaldehyde in 0.1% PBS that was kept at 3-5 °C. Brains removed from the animals were incubated for 1 hour in 4% formaldehyde in PBS and then placed in 10% sucrose solution. 50 μm coronal DBB brain slices were cut using a cryostat and then blocked with 1% bovine serum albumin for 1 hour. Slices were then incubated overnight with primary rabbit antibodies for vesicular choline acetyltransferase at 3-5 °C (vChAT; 1:2500 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, USA). Slices were then washed 3 times in PBS and incubated with Alexa 488 chicken anti-rabbit antibodies for 1.5 hours to yield green fluorescence (1:400 dilution) and washed 3 times in PBS. Slides were allowed to air dry and dehydrated with ethanol and xylene before being coverslipped with Cytoseal (Canada

Wide Scientific, Ottawa, Canada). Slides were examined with a Zeiss Axioplan 2 and images captured with Zeiss Axiocam MRc camera and MGrab software.

Drugs

$A\beta_{1-42}$ and $A\beta_{42-1}$ were purchased from American Peptide Co. (Sunnyvale, USA). $A\beta_{1-42}$ was freshly prepared on the day of the experiment from aliquots stored at – 80 °C. All voltage clamp experiments were conducted in the presence of 1 μM tetrodotoxin (TTX) and either 10 μM bicuculline or 50 μM picrotoxin. All chemicals were purchased from Sigma except for the following. TTX was purchased from Alamone Lab (Jerusalem, Israel). All drugs were bath applied through four-way valve system and the average time to the onset of drug action was 30 sec.

RESULTS

Cy3-192 IgG Labeling Colocalizes with vChAT Labeling in DBB Neurons

Immunohistochemical experiments demonstrated significant co-localization of Cy3-192 IgG labeling with vChAT labeling (Figures 3-2A, B), confirming previous studies suggesting Cy3-192 IgG-labeled cells are indeed cholinergic (Hartig et al., 1998b; Wu et al., 2000). Figure 3-2C shows a Cy3-192 IgG labeled cell viewed under a water immersion lens at 60x magnification and Figure 3-2D shows the same neuron being patched under differential infrared contrast imaging. The average capacitance of neurons was 18.9 ± 0.5 pF and the average access resistance was 6.6 ± 0.2 mΩ (n = 50). The average conductance of the cells was 2.85 ± 0.25 nS (n = 50). No significant change was observed in the conductance in any of the treatments recorded.

CNQX Abolishes mEPSCs in DBB Neurons

The character of these mEPSCs was examined with the AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). In the presence of 1 μM TTX and 50 μM picrotoxin, 2 μM CNQX abolished all synaptic activity in 3 electrophysiologically identified cholinergic neurons (Fig. 3-3). These data demonstrate that the recorded events were glutamatergic events mediated by AMPA and kainate receptors.

Aβ Modulates Frequency of mEPSCs in Cholinergic DBB Neurons

In 12 Cy3-IgG 192 labeled neurons and 3 electrophysiologically identified cholinergic neurons, bath application of 100 nM $A\beta_{1-42}$ increased mEPSC frequency in 54% of neurons tested (15/28 neurons tested; Figure 3-4A; $p<0.05$). KS testing of individual cells demonstrated that in all 15 neurons, there was no significant change in amplitude, while the interevent interval was significantly decreased ($p<0.0005$; Figure 3-4B, C). The average mEPSC frequency was 0.89 ± 0.16 Hz, which increased to 1.21 ± 0.28 Hz in the presence of 100 nM $A\beta_{1-42}$ and recovered to 0.92 ± 0.28 Hz which represents a 29.6 ± 5.4% increase in frequency ($p<0.05$; Fig. 3-4A, 3-5). The peak amplitude of mEPSCs did not exhibit any significant change in these neurons as the control peak amplitude was – 37.1 ± 1.7 pA and the peak amplitude in the presence of 100 nM $A\beta_{1-42}$ was – 36.8 ± 1.9 pA (Fig. 3-4B, 3-5). Examination of specific kinetic parameters of the mEPSCs revealed that there were no significant changes in rise time, decay time, and area between control conditions and 100 nM $A\beta_{1-42}$ treatment (Table 3-1). Linear regression of the current from -110 mV to -60 mV revealed that there were no significant difference between control conductance, which was 3.08 ± 0.32 nS and

conductance in the presence of 100 nM $A\beta_{1-42}$, which was 3.19 ± 0.45 nS (p>0.05). In 7 Cy3-192IgG labeled neurons and 2 electrophysiologically identified cholinergic neurons, bath application of 100 nM $A\beta_{1-42}$ significantly reduced mEPSC frequency from 0.97 ± 0.21 to 0.68 ± 0.18 Hz, which represents a 31.8 ± 4.8% decrease of frequency (Figure 3-6 and 3-7; p<0.05; 9/28 neurons). In all 9 neurons, no change in peak amplitude was observed with KS testing, however, there was a significant decrease in the interevent interval (p<0.0005). The peak amplitude was -34.7 ± 2.7 pA under control conditions, while in the presence of 100 nM $A\beta_{1-42}$ the peak amplitude was -31.4 ± 2.3 pA (Figure 3-7B; p>0.05; n = 9). Examination of specific kinetic parameters of the mEPSCs revealed that there were no significant changes in rise time (10%-90%), decay time (90%-10%), and area between control conditions and 100 nM $A\beta_{1-42}$ treatment of cholinergic neurons (Table 3-2). In 14% of neurons (n = 4), no change in peak amplitude or frequency of 100 nM $A\beta_{1-42}$ was observed. Comparison of neurons did not reveal any difference in amplitude, frequency, or kinetic parameters between neurons that demonstrated an increase in mEPSC frequency and those that demonstrated a decrease in mEPSC frequency.

Application of the reverse peptide $A\beta_{42-1}$ did not significantly change either the peak amplitude or mEPSC frequency in Cy3 IgG192 labeled neurons (p<0.05; n = 4; Figure 3-8). In these 4 neurons, KS testing did not detect a change in either peak amplitude or interevent interval during the application of $A\beta_{42-1}$. In neurons that exhibited increased mEPSC frequency in response to $A\beta_{1-42}$, application of 100 nM $A\beta_{42-1}$ did not elicit any change in either frequency or peak amplitude. In the presence of the reverse peptide 100 nM $A\beta_{42-1}$, the frequency was 0.69 ± 0.27 Hz, which was not

significantly different from the frequency under control conditions, which was 0.71 ± 0.29 Hz (p>0.05; Figure 3-9A; n = 4). The average peak amplitude under control conditions was -41.1 ± 3.9 pA, while in the presence of $A\beta_{42\text{-}1}$, the peak amplitude was -40.0 ± 5.6 pA (p>0.05; Figure 3-9B; n = 4). No significant differences in kinetic parameters were observed between control conditions and application of 100 nM $A\beta_{42\text{-}1}$.

Aβ Mediated Increase in mEPSC Frequency is Inhibited by DHβE

The effect of the α4β2 nAChR selective antagonist DHβE on $A\beta_{1\text{-}42}$ mediated increases in mEPSC frequency was examined. Application of 100 nM $A\beta_{1\text{-}42}$ to 5 Cy3 IgG 192 labeled neurons resulted in an increase in frequency from 0.64 ± 0.13 Hz under control conditions to 0.84 ± 0.15 Hz in the presence of 100 nM $A\beta_{1\text{-}42}$, which is a 25.4 ± 7.9% increase (Figure 3-10A; p<0.05;n = 5). As previously observed, there was no significant change in peak amplitude which was -37.4 ± 1.7 pA under control conditions and -36.2 ± 0.09 pA in the presence of 100 nM $A\beta_{1\text{-}42}$. Subsequent application of 100 nM $A\beta_{1\text{-}42}$ in the presence of 10 μM DHβE blocked the increase by 100 nM $A\beta_{1\text{-}42}$. Prior to co-application of 100 nM $A\beta_{1\text{-}42}$ and 10 μM DHβE, the frequency was 0.65 Hz ± 0.17 Hz. In the presence of 100 nM $A\beta_{1\text{-}42}$ and 10 μM DHβE, the frequency was 0.67 ± 0.17 Hz, which represents a 2.4 ± 5.6% change (p>0.05; n = 5; Figure 3-10B). Under control conditions, the average peak amplitude was -36.0 ± 0.07 pA, while in the presence of 100 nM $A\beta_{1\text{-}42}$ and 10 μM DHβE, the average peak amplitude was -35.6 ± 1.8 pA (p>0.05; n = 5). Additionally, no significant change in kinetic parameters was observed (Table 3-3). KS testing of the individual neurons also demonstrated that $A\beta_{1\text{-}42}$ mediated increases of mEPSCs were inhibited by DHβE (Figure 3-11).

Control experiments demonstrated that $A\beta_{1-42}$ mediated increases of frequency in electrophysiologically identified cholinergic neurons were not time dependent as in 3 cholinergic neurons, consecutive application of 100 nM $A\beta_{1-42}$ 7 minutes apart yielded significant increases of mEPSC frequency of 27.3 ± 4.5% and 29.8 ± 3.5% ($p>0.05$). There was no significant change in peak amplitude. KS testing of the individual neurons also showed that the effect of $A\beta_{1-42}$ was not time dependent. Application of 10 μM DHβE on electrophysiologically identified cholinergic neurons did not significantly change mEPSC frequency or peak amplitude when examined with KS testing ($p>0.0005$; n = 2).

Aβ Mediated Decrease in mEPSC Frequency Persists in the Presence of DHβE

In Cy3-192 IgG labeled neurons, 10 μM DHβE did not block the $A\beta_{1-42}$ mediated decrease in frequency. In the neurons tested, application of $A\beta_{1-42}$ on its own significantly decreased frequency from 1.75 ± 0.51 Hz to 1.25 ± 0.36 Hz, which represents a 27.5 ± 3.7% decrease (Figure 3-12; n = 5, $p<0.05$). As previously described, there was no significant change in the average peak amplitude in the presence of 100 nM $A\beta_{1-42}$. Subsequent application of 100 nM $A\beta_{1-42}$ and 10 μM DHβE did not significantly inhibit the decreased frequency observed. The average frequency under control conditions was 1.50 ± 0.45 Hz and in the presence of 100 nM $A\beta_{1-42}$ and 10 μM DHβE, the frequency decreased to 1.15 ± 0.37 Hz, representing a 24.5 ± 2.8% decrease ($p<0.05$; n = 5). After washout, the frequency recovered to 1.46 ± 0.46 Hz. The peak amplitude was -31.5 ± 4.2 pA under control conditions and -32.4 ± 3.5 pA in the presence of 100 nM $A\beta_{1-42}$ and 10 μM DHβE ($p<0.05$; n = 5). Additionally, no changes to kinetic parameters were observed between treatments (Table 3-4). KS testing of the individual

neurons also demonstrated that $A\beta_{1-42}$ mediated decreases of mEPSCs were not inhibited by DHβE (Figure 3-13).

Nicotine Increases mEPSC Frequency in a Dose-Dependent Manner

To further examine nicotinic receptors on cholinergic basal forebrain neurons, various doses of nicotine were bath applied to Cy3-192IgG labeled neurons. Application of 1 μM (n = 7), 10 μM (n = 5), and 100 μM (n = 5) nicotine revealed that Cy3-192IgG labeled neurons exhibited a dose-dependent increase in frequency in the majority of neurons (19/22) (Fig. 3-14A, B). mEPSC frequency increased from 0.66 ± 0.19 Hz to 1.02 ± 0.25 Hz in the presence of 100 μM nicotine and recovered back to 0.71 ± 0.23 Hz after washout. No significant changes in amplitude, rise time, decay time or area were observed (Tables 3-5 to 3-7). In 3 of 9 neurons, bath application of 100 μM nicotine did not significantly change mEPSC frequency. No significant changes in amplitude, decay time or area were observed, however there was a significant increase in rise time (Table 3-8). KS testing of individual neurons, demonstrated that in all 9 neurons, there was no change in peak amplitude during the application of 100 μM nicotine (Figure 3-15). In 6 of 9 neurons, KS testing demonstrated that 100 μM nicotine significanty decreased the interevent interval ($p<0.0005$), while in 3 of 9 neurons, KS testing did not show any change in the interval event interval ($p<0.0005$).

Acetylcholine Increases mEPSC Frequency in a Dose-Dependent Manner

In the next set of experiments, the endogenous neurotransmitter ACh in the presence of 1 μM atropine, a muscarinic receptor blocker, was applied to Cy3-192 IgG labeled neurons. Application of ACh demonstrated, in a dose-dependent manner, an increase in mEPSC frequency (Figure 3-15). For 1 mM of ACh, mEPSC frequency

increased from 0.49 ± 0.03 Hz to 0.76 ± 0.05 Hz and then recovered back to 0.44 ± 0.05 Hz, which represents an average increase of 54 ± 12%. No significant changes to peak amplitude, 90%-10% decay time or area were observed (Tables 3-9 to 3-11). KS testing of individual neurons demonstrated that 1 mM ACh significantly decreases interevent interval in cholinergic neurons ($p<0.0005$) without significantly changing peak amplitude.

DISCUSSION

In this study, the effect of $A\beta_{1-42}$ and nicotine on fluorescently labeled cholinergic DBB neurons were examined. In the majority of cholinergic neurons, bath applications of $A\beta_{1-42}$ significantly increased the frequency of mEPSCs without any significant change in amplitude or kinetic parameters of EPSCs. Furthermore, mEPSCs recorded from DBB neurons were mediated by the AMPA and kainate subtypes of glutamate receptors as application of CNQX, an AMPA/kainate receptor antagonist, completely abolished the spontaneous mEPSCs. This effect of $A\beta_{1-42}$ could be blocked by a non-$\alpha7$, $\alpha4\beta2$ selective nAChR antagonist, suggesting that soluble $A\beta_{1-42}$ is able to modulate presynaptic glutamatergic neurotransmission via activation of $\alpha4\beta2$ nAChRs.

In another subset of cholinergic neurons, $A\beta_{1-42}$ decreased the frequency of mEPSCs without changing any kinetic parameters. The decrease in frequency was not inhibited by DHβE suggesting that $A\beta_{1-42}$ is mediating a presynaptic effect via a different receptor. No differences in kinetic parameters could be observed between the two populations of neurons.

Applications of nicotine and ACh (in the presence of atropine) resulted in a dose-dependent increase in frequency, without a significant change in amplitude of the mEPSCs that mimicked the effects of $A\beta_{1-42}$.

Soluble Aβ Modulates Synaptic Transmission onto Cholinergic DBB Neurons

There is significant controversy regarding the interaction of $A\beta_{1-42}$ and nAChRs. Initial binding studies demonstrated that $A\beta_{1-42}$ binds to both α7 nAChRs with picomolar affinity and α4β2 nAChRs with nanomolar affinity, although these studies have been difficult to replicate in other laboratories (Wang et al., 2000b; Wang et al., 2000a). Nonetheless, a number of electrophysiological studies have demonstrated that $A\beta_{1-42}$ can exert its effects through both α7 and α4β2 nAChRs in a variety of *in vitro* preparations (Pettit et al., 2001; Dineley et al., 2002; Fu and Jhamandas, 2003). However, the effect of $A\beta_{1-42}$ interaction with the nAChR remains controversial as some studies suggest that $A\beta_{1-42}$ activates α4β2 nAChRs, while other studies suggest that $A\beta_{1-42}$ inhibits nAChRs (Fu and Jhamandas, 2003; Wu et al., 2004). In this study, $A\beta_{1-42}$ was demonstrated to mediate an increase in the frequency of spontaneous glutamatergic mEPSCs in a subset of cholinergic DBB neurons via activation of non-α7 nAChR, which is probably of the α4β2 nAChR subtype. This $A\beta_{1-42}$ activation of α4β2 nAChR is in agreement with single channel recordings from DBB neurons which demonstrated that $A\beta_{1-42}$ was able to act as an agonist at α4β2 nAChR in DBB neurons (Fu and Jhamandas, 2003). However, the effects of $A\beta_{1-42}$ observed in this study were localized to a presynaptic locus of action, whereas Fu and Jhamandas (2003) reported post-synaptic α4β2 nAChR-mediated responses. This difference may reflect the topographical differences in the location of glutamate terminals and nAChRs in that post-synaptic nAChRs may not be in close

proximity to glutamatergic presynaptic terminals in order to mediate a recordable effect. Alternately, mEPSC recordings may not be sensitive enough to detect the level of change in the kinetic parameters that could result in subtle post-synaptic modulation mediated by nAChRs.

In another subset of cholinergic neurons, DHβE did not affect the $A\beta_{1-42}$-induced inhibition of mEPSC frequency, suggesting that such effects of $A\beta_{1-42}$ are mediated by another mechanism. As there are a plethora of receptors in the basal forebrain and the cholinergic neurons receive innervation from many different areas of the brain, further experiments need to be conducted in order to elucidate the possible receptor that could mediate this aspect of synaptic modulation.

Activation of nAChRs Modulate Synaptic Transmission onto Cholinergic DBB Neurons

These experiments demonstrated that both nicotine and ACh in the presence of atropine were able to modulate spontaneous mEPSCs via a presynaptic mechanism in a dose dependent manner. The increase in mEPSC frequency without any change in peak amplitude observed is similar to that observed to the effect that $A\beta_{1-42}$ had on a population of neurons. Studies in other areas of the brain such as the cortex, hippocampus, and striatum have demonstrated that presynaptic nAChRs can modulate synaptic transmission (Alkondon et al., 1997; Girod et al., 2000; Cao et al., 2005). These studies have examined nAChR modulation of dopamine, GABA, and glutamate release and found that activation of both α7 and α4β2 nAChR on presynaptic terminals can increase neurotransmitter release.

Studies examining nicotine in the basal forebrain neurons did not reveal any changes in cholinergic neuron firing in the presence of ACh or nicotine (Wu et al., 2000;

Wu et al., 2003). However, one recent study demonstrated that nAChRs could modulate the excitability of cholinergic DBB neurons via α4β2 and α7 nAChRs (Henderson et al., 2005). We were unable to observe any changes in conductance or in the kinetic parameters of mEPSCs to suggest that there was post-synaptic modulation of the glutamate response by nicotine or ACh.

Future Experiments

Two key issues regarding nAChRs need to be resolved. First, the identity of the receptor involved in $A\beta_{1-42}$ decreases of mEPSCs needs to be examined. This decrease may be mediated by $A\beta_{1-42}$ effects on another neurotransmitter system in the DBB or possibly by the α7 nAChR. An experiment the α7 nAChR antagonist αBTX was applied in the presence of Aβ would resolve this issue. Secondly, pharmacological experiments need to be performed on DBB neurons to elucidate if α4β2 or α7 nAChRs are involved in mediating the effects nicotine and ACh. Furthermore, it would be of interest to examine the link between Aβ and nicotine effects on synaptic function. Experiments in which Aβ and nicotine were applied concurrently after application of a single dose would elicit whether Aβ and nicotine were acting on the same receptor to mediate their effects on mEPSCs.

Functional Implications

This study demonstrates that $A\beta_{1-42}$ is capable of modulating synaptic transmission in septo-hippocampal neurons of the basal forebrain, an important pathway for learning and memory via α4β2 nAChRs in cholinergic neurons of the basal forebrain. Thus the implications of this research are twofold. First, this study suggests that modulation of synaptic transmission by soluble forms of $A\beta_{1-42}$ may be a potential

pathological mechanism for the cognitive decline observed early on in AD. $A\beta_{1-42}$ augmentation of glutamate neurotransmission could lead to excitotoxicity over the long term, while $A\beta_{1-42}$ reduction of glutamate neurotransmission, would weaken cholinergic tone and lead to impaired cognition. Secondly, as the detection of AD becomes more sensitive, the nAChR system is a potential therapeutic target in the treatment of AD.

Table 3-1. Kinetic Parameters of Cholinergic Neurons that Demonstrate an Increase of mEPSCs in the Presence of 100 nM $A\beta_{1-42}$.

Statistic	Control	100 nM $A\beta_{1-42}$	Recovery
Peak Amplitude (pA)	-37.1 ± 1.7	-36.8 ± 1.9	-35.9 ± 1.7
10%-90% Rise time (ms)	0.73 ± 0.09	0.80 ± 0.10	0.81 ± 0.10
90%-10% Decay time (ms)	2.84 ± 0.80	2.88 ± 0.31	2.86 ± .32
Area (nA ms)	-0.068 ± 0.007	-0.068 ± 0.007	-0.068 ± 0.007

Table 3-2. Kinetic Parameters of Cholinergic Neurons that Demonstrate a Decrease of mEPSCs in the presence of 100 nM $A\beta_{1-42}$.

Statistic	Control	100 nM $A\beta_{1-42}$	Recovery
Peak Amplitude (pA)	-35.0 ± 2.9	-31.4 ± 2.3	-33.3 ± 2.7
10%-90% Rise time (ms)	0.65 ± 0.04	0.64 ± 0.06	0.63 ± 0.06
90%-10% Decay time (ms)	2.56 ± 0.34	2.53 ± 0.34	2.51 ± 0.31
Area (nA ms)	-0.063 ± 0.006	-0.056 ± 0.006	-0.058 ± 0.006

Table 3-3. Kinetic Parameters of Cholinergic Neurons that Demonstrate an Increase in the Frequency of mEPSCs in the Presence of 100 nM $A\beta_{1-42}$ that are Subsequently Exposed to 100 nM $A\beta_{1-42}$ and 10 μM DHβE

Statistic	Control	100 nM $A\beta_{1-42}$ + 10 μM DHβE	Recovery
Peak Amplitude (pA)	-36.0 ± 0.7	-35.6 ± 0.2	-34.8 ± 0.9
10%-90% Rise time (ms)	0.77 ± 0.06	0.80 ± 0.07	0.78 ± 0.06
90%-10% Decay time (ms)	2.27 ± 0.17	2.28 ± 0.23	2.26 ± 0.20
Area (nA ms)	-0.052 ± 0.007	-0.053 ± 0.007	-0.053 ± 0.007

Table 3-4. Kinetic parameters of Cholinergic Neurons that Demonstrate a Decrease in the Frequency of mEPSCs in the presence of 100 nM $A\beta_{1-42}$ that are Subsequently Exposed to 100 nM $A\beta_{1-42}$ and 10 μM DHβE

Statistic	Control	100 nM $A\beta_{1-42}$ + 10 μM DHβE	Recovery
Peak Amplitude (pA)	-33.3 ±4.5	-32.7 ± 4.1	-31.5 ± 4.2
10%-90% Rise time (ms)	0.67 ± 0.04	0.66 ± 0.05	0.69 ± 0.05
90%-10% Decay time (ms)	1.77 ± 0.36	1.69 ± 0.39	2.08 ± 0.17
Area (nA ms)	-0.054 ± 0.007	-0.051 ± 0.006	-0.051 ± 0.007

Table 3-5. Kinetic Parameters of Cholinergic Neurons that Demonstrate an Increase in the Frequency of mEPSCs in response to Bath Application of 100 μM Nicotine.

Statistic	Control	100 μM Nicotine	Recovery
Peak Amplitude (pA)	-27.3 ± 4.6	-22.6 ± 2.5	-24.0 ± 4.0
10%-90% Rise time (ms)	0.62 ± 0.07	0.72 ± 0.07	0.74 ± 0.08
90%-10% Decay time (ms)	2.12 ± 0.19	1.99 ± 0.17	2.31 ± 0.22
Area (nA ms)	-0.047 ± 0.006	-0.041 ± 0.04	-0.045 ± 0.006

Table 3-6. Kinetic Parameters of Cholinergic Neurons Demonstrating an Increase of mEPSC Frequency in Response to Bath Application of 10 μM Nicotine. * denotes (p<0.05)

Statistic	Control	10 μM Nicotine	Recovery
Peak Amplitude (pA)	-27.4 ± 5.7	-27.2 ± 4.5	-25.7 ± 5.4
10%-90% Rise time (ms)	0.61 ± 0.05	0.93 ± 0.20*	0.98 ± 0.23
90%-10% Decay time (ms)	2.03 ± 0.16	1.99 ± 0.19	2.07 ± 0.16
Area (nA ms)	-0.054 ± 0.001	-0.051 ± 0.010	-0.048 ± 0.010

Table 3-7. Kinetic Parameters of Cholinergic Neurons Demonstrating an Increase of mEPSC Frequency in Response to Bath Application of 1 μM Nicotine. * denotes (p<0.05)

Statistic	Control	10 μM Nicotine	Recovery
Peak Amplitude (pA)	-27.3 ± 3.2	-26.8 ± 2.6	-27.6 ± 2.6
10%-90% Rise time (ms)	0.60 ± 0.07	0.62 ± 0.06	0.61 ± 0.05
90%-10% Decay time (ms)	2.06 ± 0.16	1.91 ± 0.11	1.96 ± 0.10
Area (nA ms)	-0.050 ± 0.006	-0.049 ± 0.005	-0.05 ± 0.005

Table 3-8. Kinetic Parameters of Cholinergic Neurons Demonstrating no Change of mEPSC Frequency in Response to Bath Application of 100 μM Nicotine. * denotes (p<0.05)

Statistic	Control	100 μM Nicotine	Recovery
Peak Amplitude (pA)	-29.9 ± 4.6	-29.7 ± 5.0	-26.9 ± 2.2
10%-90% Rise time (ms)	0.79 ± 0.18	0.93 ± 0.20*	0.98 ± 0.23
90%-10% Decay time (ms)	2.25 ± 0.40	2.53 ± 0.38	2.49 ± 0.45
Area (nA ms)	-0.048 ± 0.03	-0.057 ± 0.02	-0.053 ± 0.02

Table 3-9. Kinetic Parameters of Cholinergic Neurons in the Presence of 1 mM ACh. * denotes (p<0.05)

Statistic	Control	1 mM ACh	Recovery
Peak Amplitude (pA)	-29.4 ± 3.5	-22.7 ± 2.8	-23.3 ± 2.4
10%-90% Rise time (ms)	0.51 ± 0.05	0.67 ± 0.08	0.70 ± 0.07
90%-10% Decay time (ms)	1.62 ± 0.12	1.60 ± 0.10	1.68 ± 0.10
Area (nA ms)	-0.043 ± 0.003	-0.034 ± 0.002	-0.036 ± 0.003

Table 3-10. Kinetic parameters of Cholinergic Neurons in the Presence of 100 μM ACh. * denotes ($p<0.05$)

Statistic	Control	100 μM ACh	Recovery
Peak Amplitude (pA)	-23.0 ± 4.4	-20.5 ± 4.8	-22.1 ± 5.8
10%-90% Rise time (ms)	0.56 ± 0.04	0.63 ± 0.05	0.57 ± 0.07
90%-10% Decay time (ms)	1.60 ± 0.17	1.52 ± 0.18	1.54 ± 0.17
Area (nA ms)	-0.035 ± 0.008	-0.032 ± 0.009	-0.032 ± 0.008

Table 3-11. Kinetic Parameters of Cholinergic Neurons in the Presence of 1 μM ACh. * denotes ($p<0.05$)

Statistic	Control	1 μM ACh	Recovery
Peak Amplitude (pA)	-25.5 ± 3.4	-23.4 ± 1.1	-24.3 ± 2.0
10%-90% Rise time (ms)	0.67 ± 0.11	0.66 ± 0.06	0.62 ± 0.03
90%-10% Decay time (ms)	1.65 ± .010	1.63 ± 0.07	1.82 ± 0.10
Area (nA ms)	-0.042 ± 0.006	-0.039 ± 0.002	-0.043 ± 0.004

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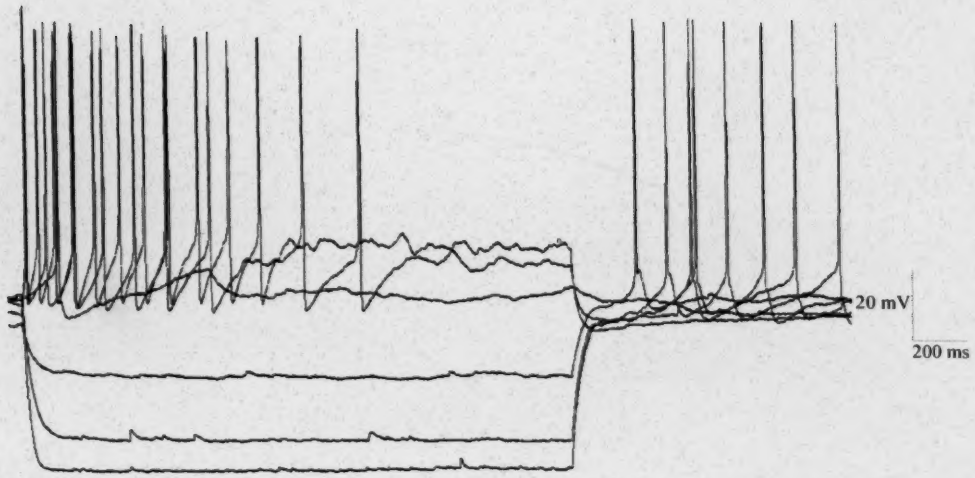

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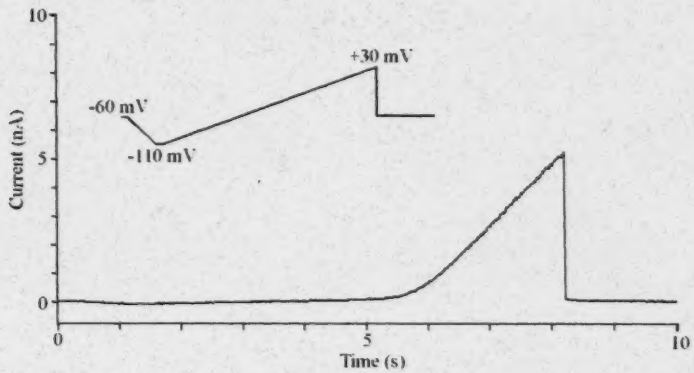

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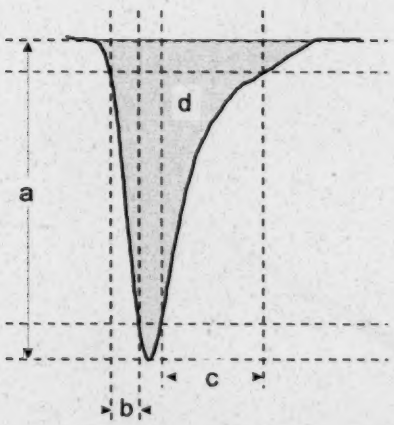
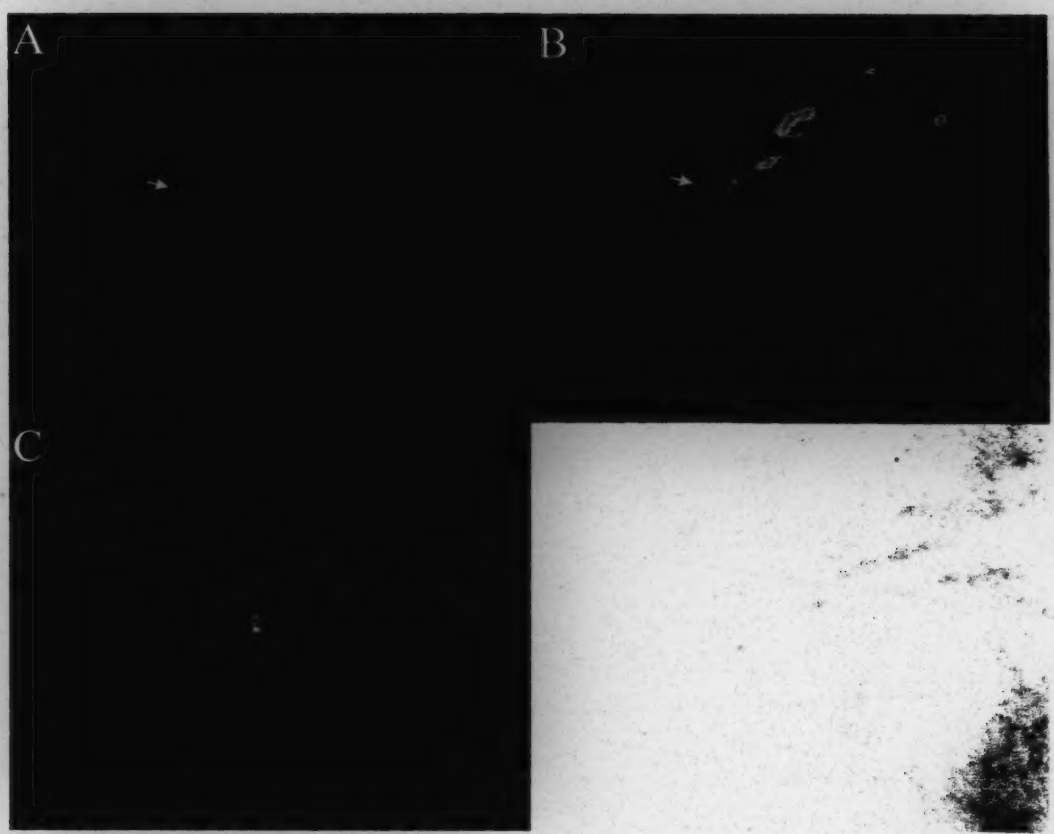
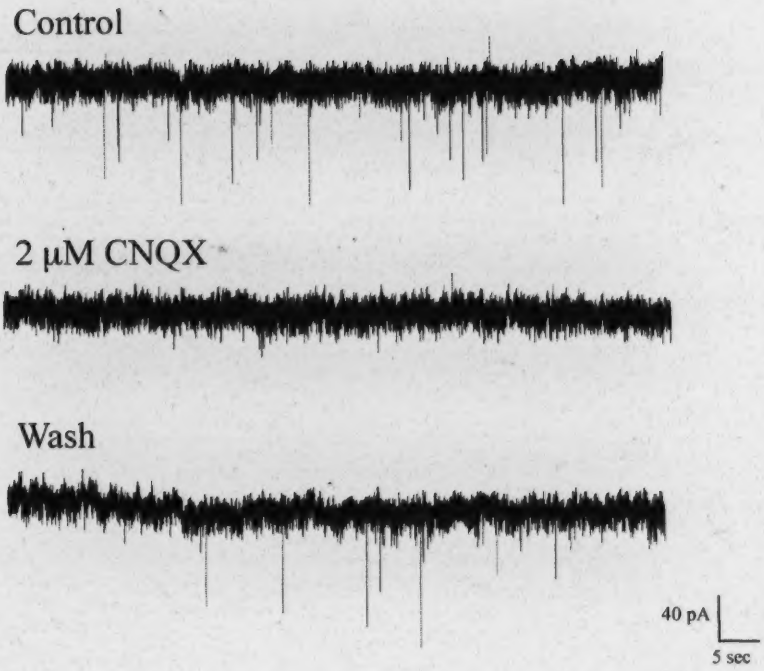



A
B
C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Wash
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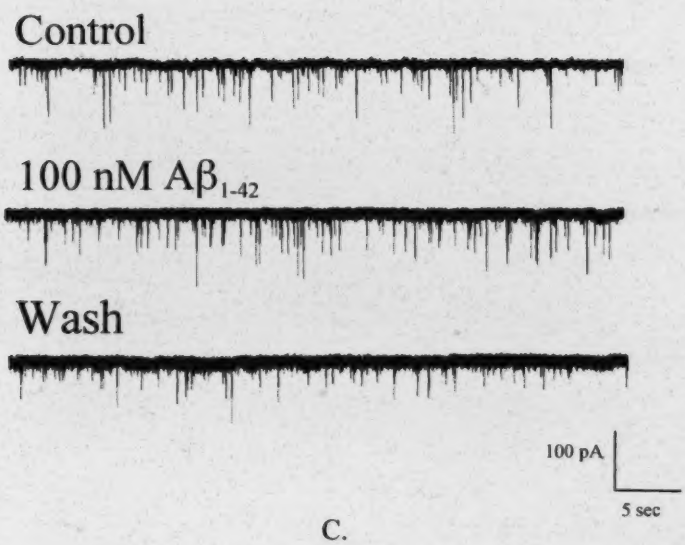
5 sec

A.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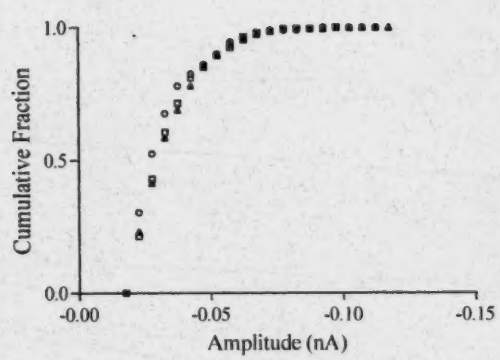
Wash
100 pA
5 sec

B.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0.0
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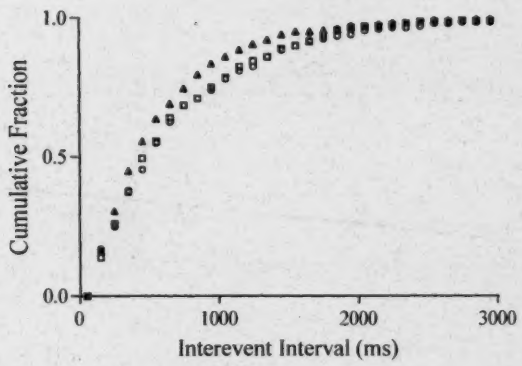
-0.05
-0.10
-0.15
Amplitude (nA)

C.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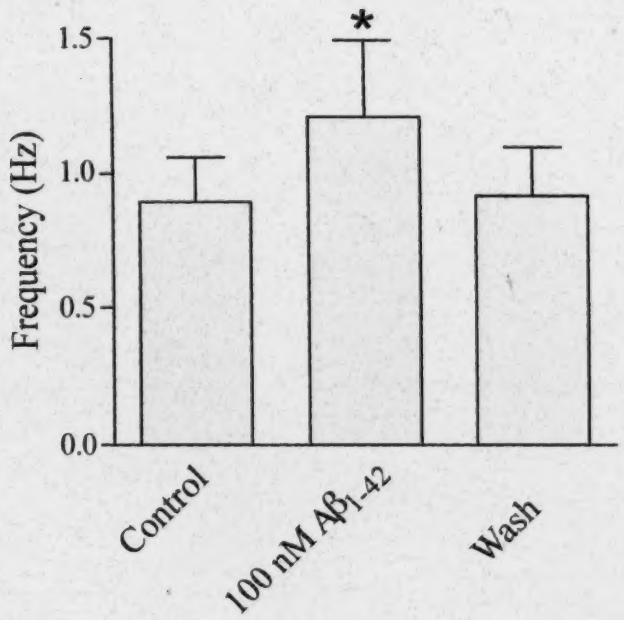
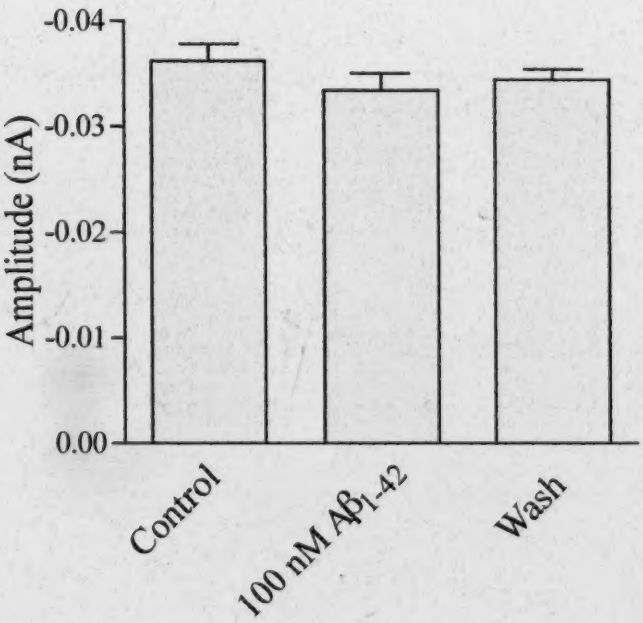
0.0
0
1000
2000
3000
Interevent Interval (ms)

A.


*
1.5
1.0
0.5
0.0
Frequency (Hz)
Control
100 nM Aβ1-42
Wash

B.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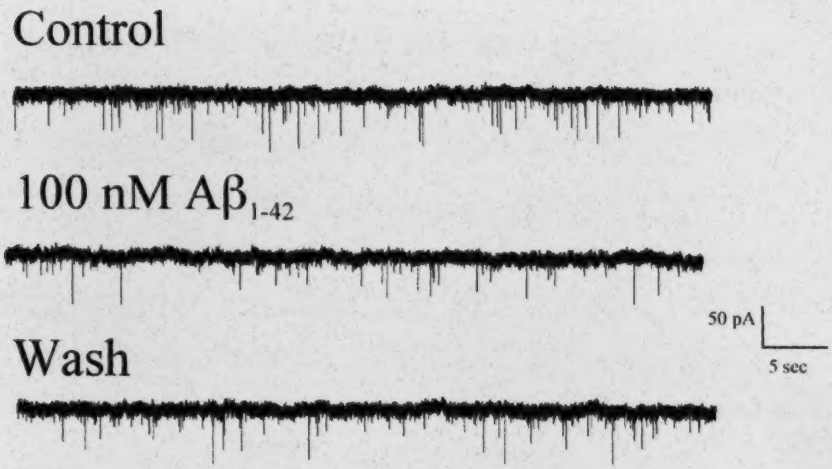
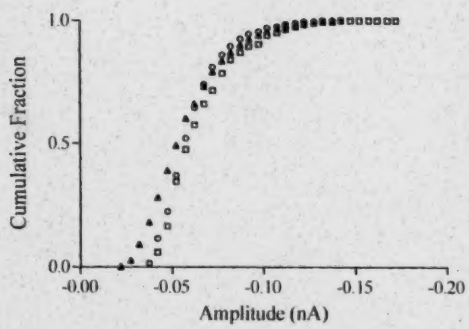
-0.03
-0.02
-0.01
0.00
Amplitude (nA)
Control
100 nM Aβ1-42
Wash

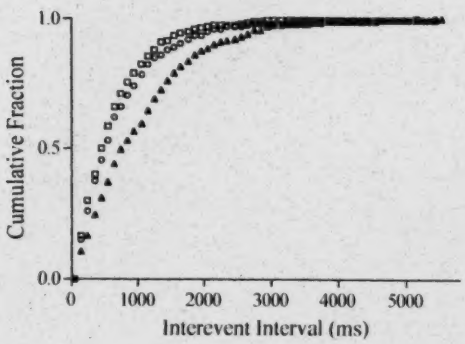
A.


Control
100 nM Aβ1-42
50 pA
5 sec
Wash

B.


Cumulative Fraction
1.0
0.5
0.0
-0.00
-0.05
-0.10
-0.15
-0.20
Amplitude (nA)

C.


Cumulative Fraction
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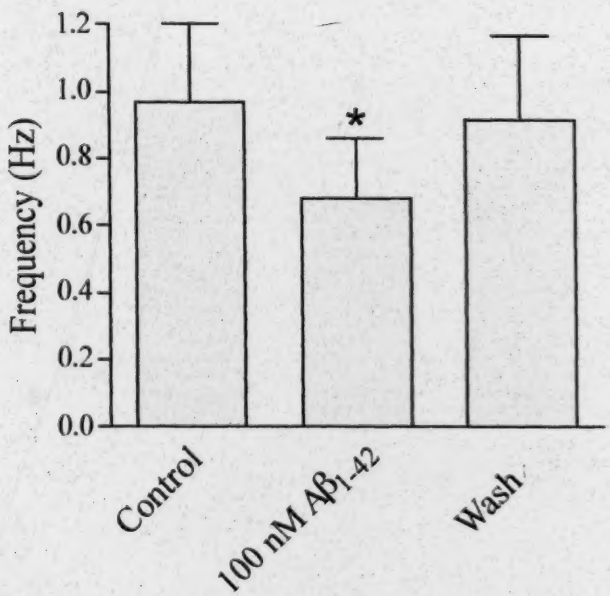
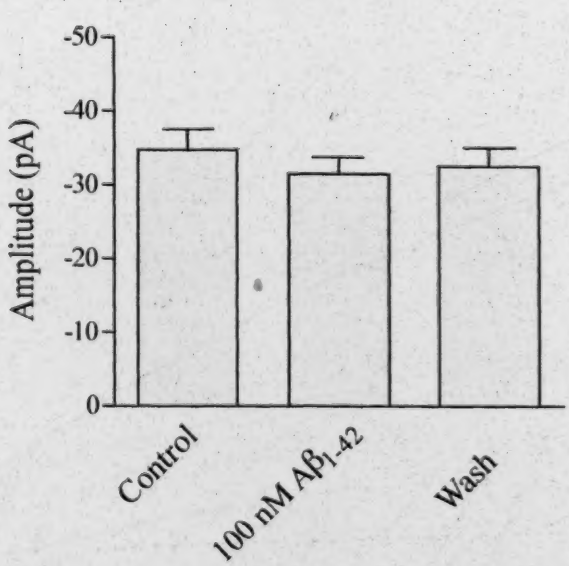
0.5
0.0
0
1000
2000
3000
4000
5000
Interevent Interval (ms)

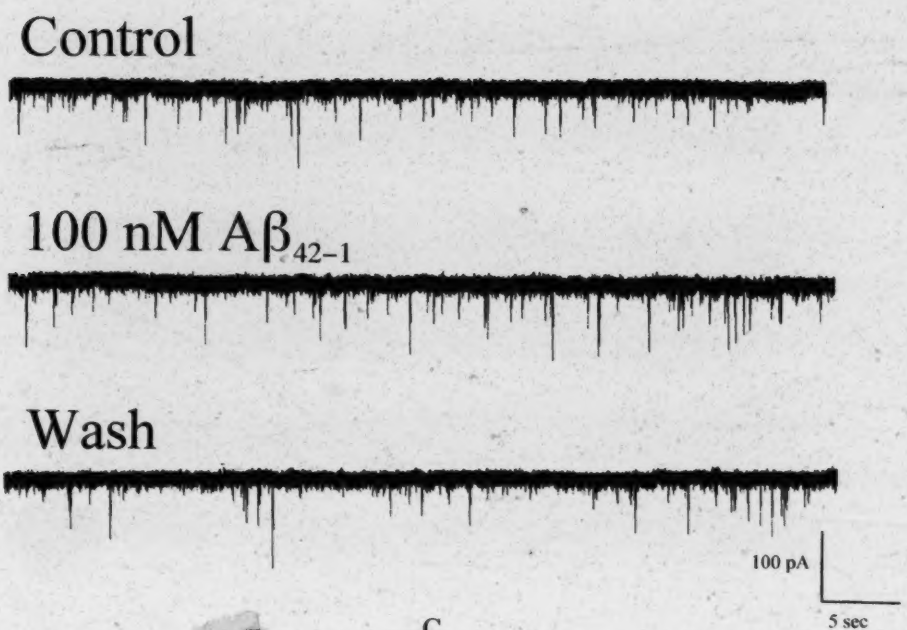
A.


1.2
1.0
0.8
0.6
0.4
0.2
0.0
Frequency (Hz)
*
Control
100 nM Aβ1-42
Wash

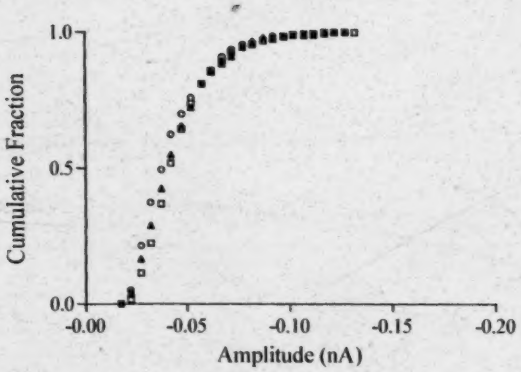
B.


-50
-40
-30
-20
-10
0
Amplitude (pA)
Control
100 nM Aβ1-42
Wash

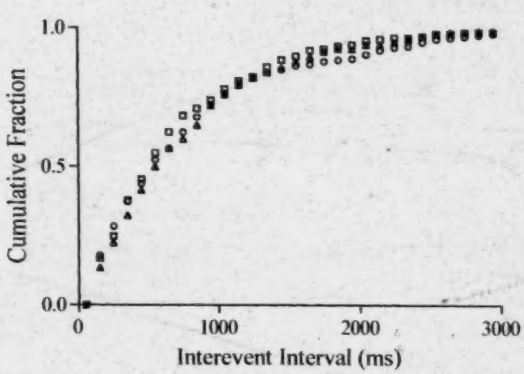
A.


Control
100 nM Aβ42-1
Wash
100 pA
5 sec

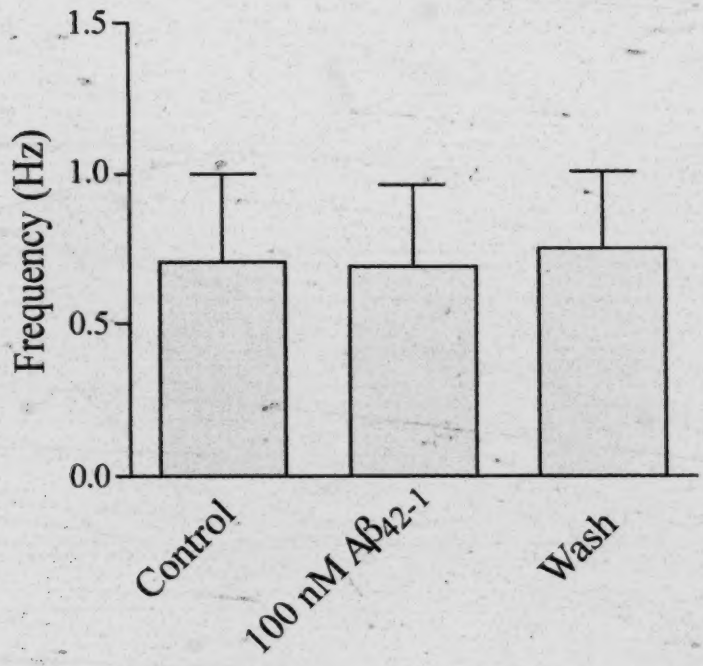
B.


Cumulative Fraction
1.0
0.5
0.0
-0.00
-0.05
-0.10
-0.15
-0.20
Amplitude (nA)

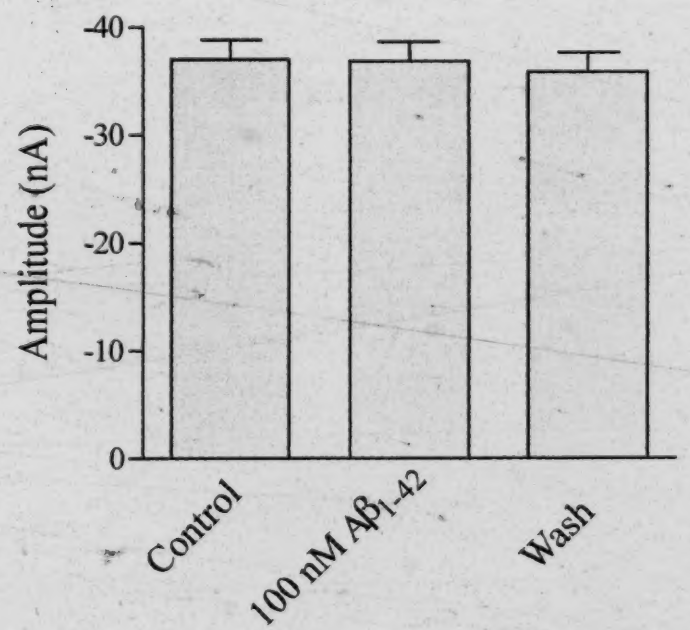
C.


Cumulative Fraction
1.0
0.5
0.0
0
1000
2000
3000
Interevent Interval (ms)

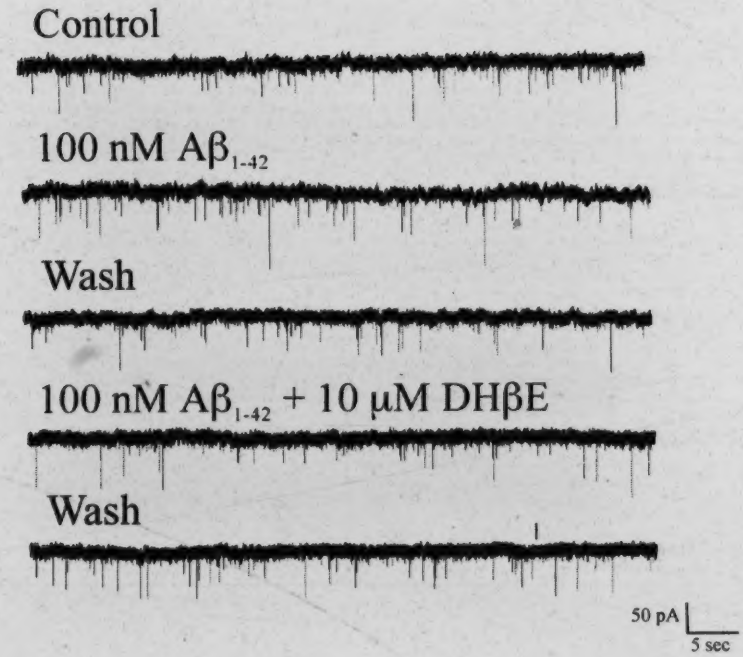
A.


1.5
1.0
0.5
0.0
Frequency (Hz)
Control
100 nM Aβ42-1
Wash

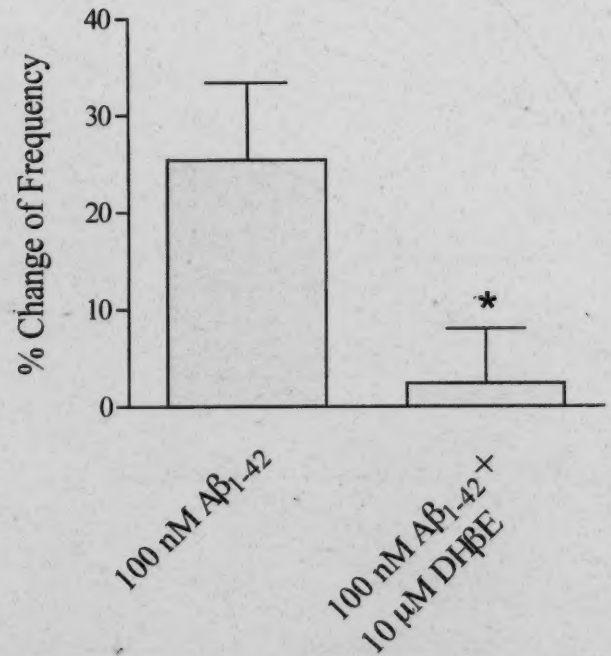
B.

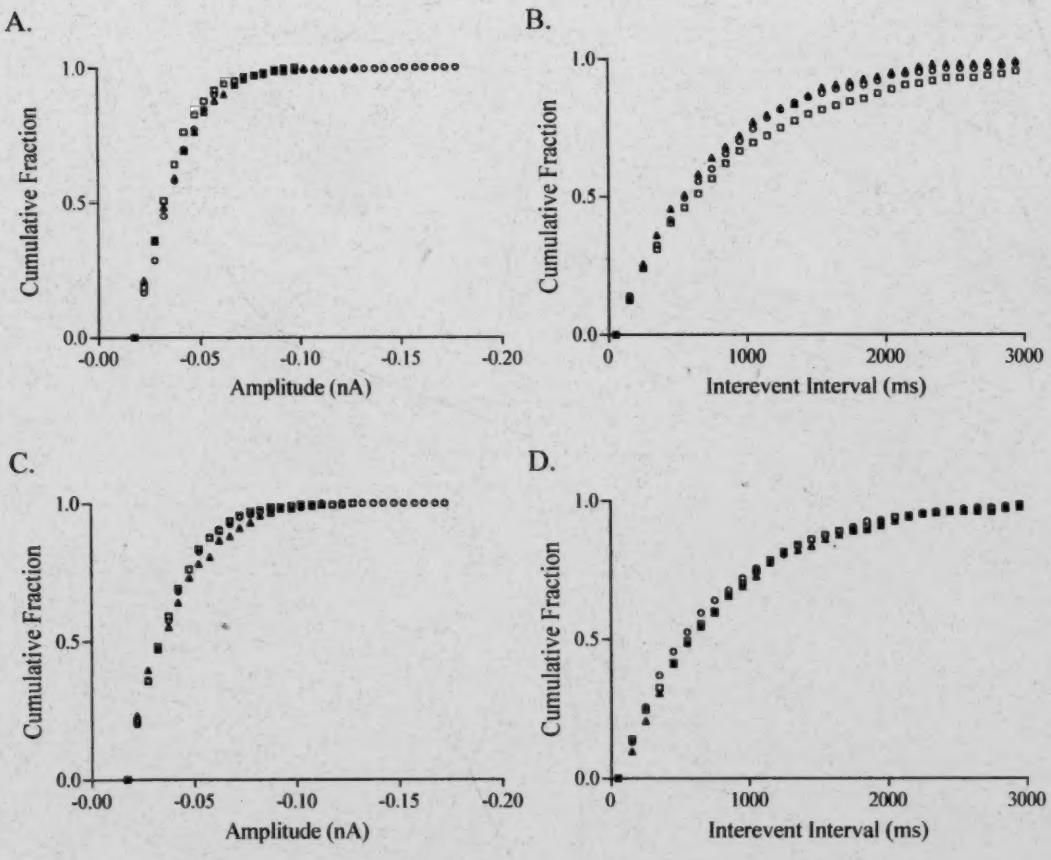

-40
-30
-20
-10
0
Amplitude (nA)
Control
100 nM Aβ1-42
Wash

A.

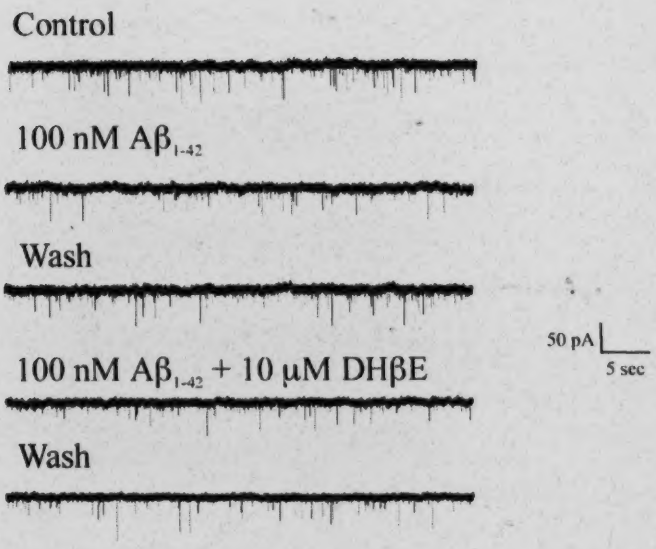

Control
100 nM Aβ1-42
Wash
100 nM Aβ1-42 + 10 μM DHβE
Wash
50 pA
5 sec

B.

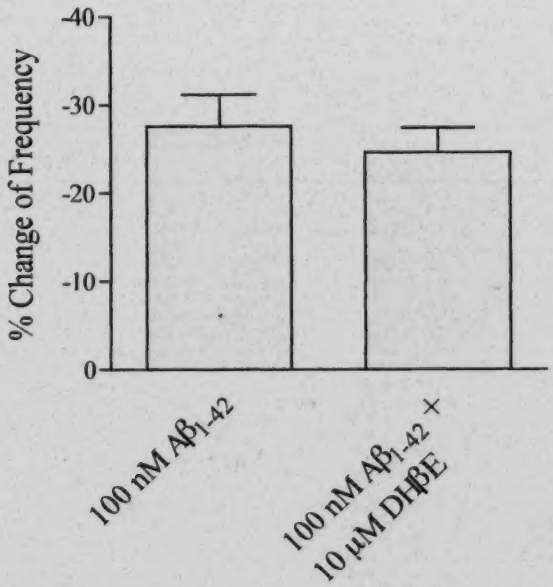

% Change of Frequency
40
30
20
10
0
*
100 nM Aβ1-42
100 nM Aβ1-42 + 10 μM DHβE

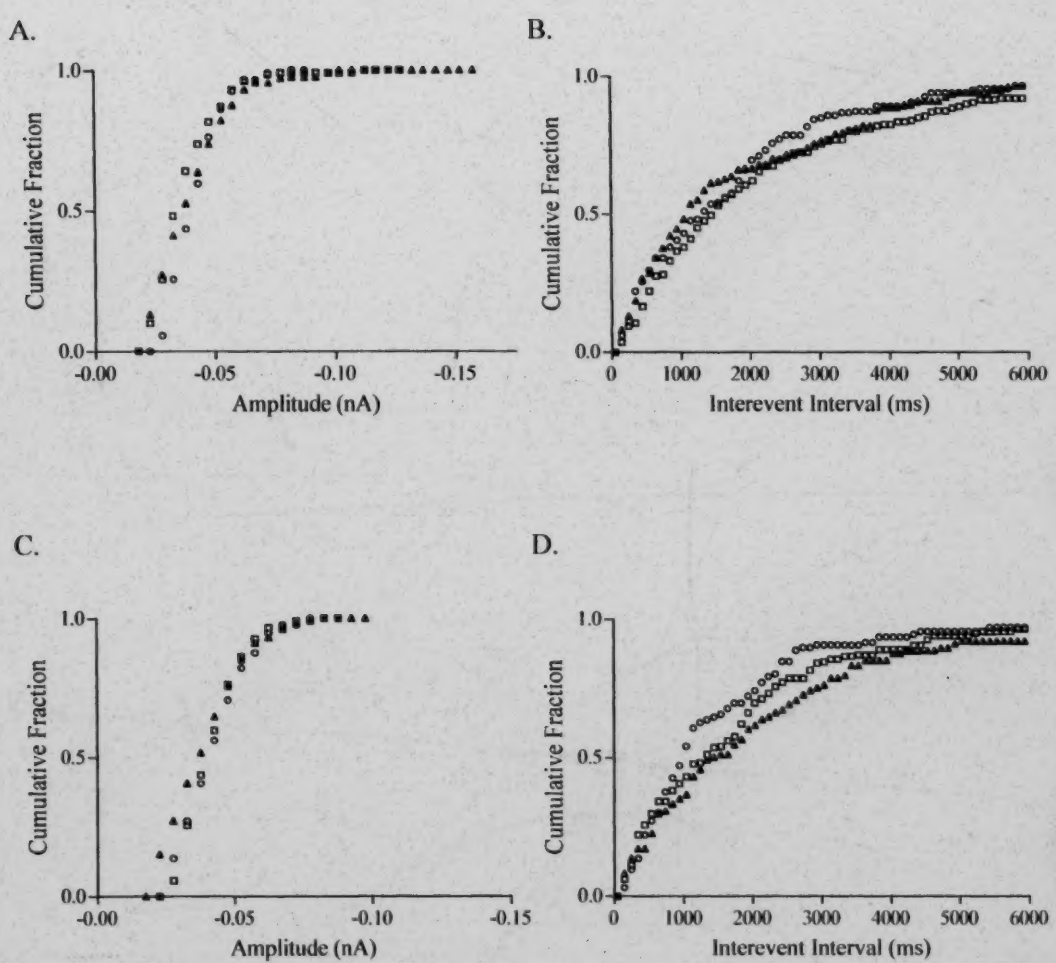

A.
B.
C.
D.
Cumulative Fraction
1.0
0.5
0.0
-0.00
-0.05
-0.10
-0.15
-0.20
Amplitude (nA)
0
1000
2000
3000
Interevent Interval (ms)

A.

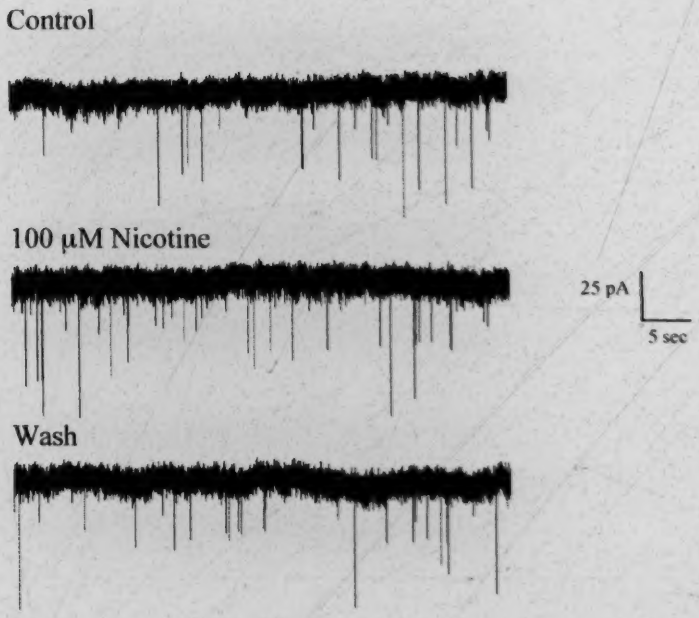

Control
100 nM Aβ1-42
Wash
50 pA
5 sec
100 nM Aβ1-42 + 10 μM DHβE
Wash

B.

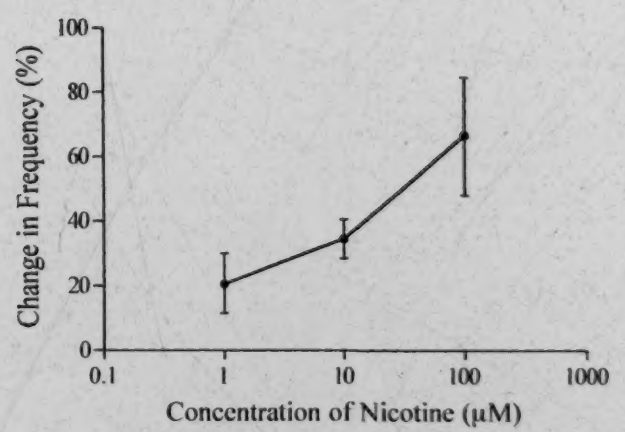

% Change of Frequency
-40
-30
-20
-10
0
100 nM Aβ1-42
100 nM Aβ1-42 + 10 μM DHβE


A.
B.
C.
D.
Cumulative Fraction
1.0
0.5
0.0
-0.00
-0.05
-0.10
-0.15
Amplitude (nA)
0
1000
2000
3000
4000
5000
6000
Interevent Interval (ms)

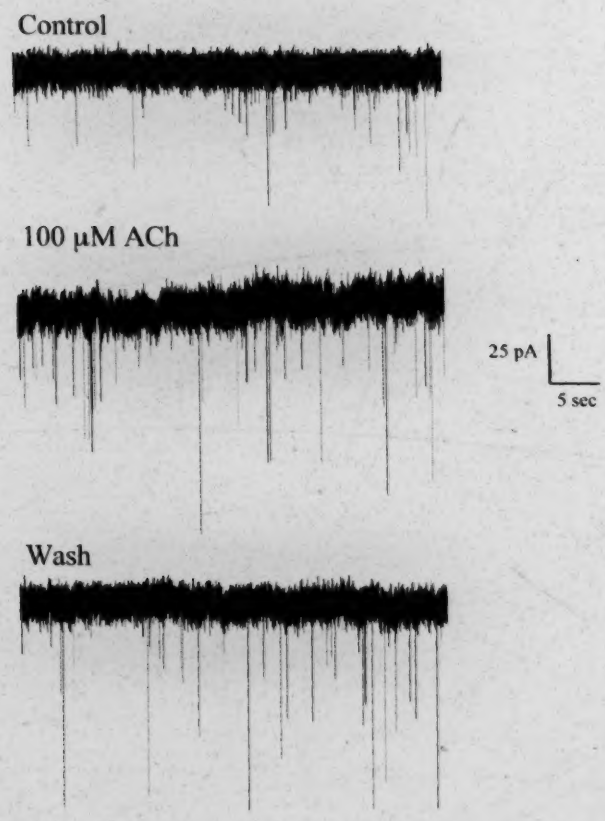
A.


Control
100 μM Nicotine
Wash
25 pA
5 sec

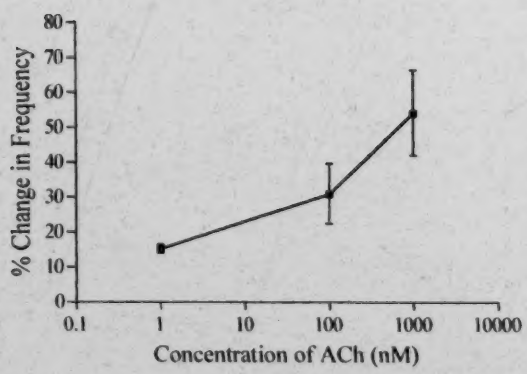
B.


Change in Frequency (%)
100
80
60
40
20
0
0.1
1
10
100
1000
Concentration of Nicotine (μM)

A.


Control
100 μM ACh
25 pA
5 sec
Wash

B.


% Change in Frequency
80
70
60
50
40
30
20
10
0
0.1
1
10
100
1000
10000
Concentration of ACh (nM)

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Chapter 4

General Discussion

This thesis demonstrates that the sum of the actions of Aβ in the cholinergic basal forebrain are expressed through nAChRs. In the first part of this study, the effect of $A\beta_{1-42}$ potentiation on repeated activation of nAChR mediated $[Ca^{2+}]_i$ rises was examined. In the second part, $A\beta_{1-42}$ was demonstrated to modulate glutamate-mediated synaptic transmission in fluorescently labeled cholinergic DBB neurons of the basal forebrain via nAChRs. As the cholinergic neurons in this area of the brain play an important role in memory and learning and undergo early neurodegeneration in AD, Aβ-nAChR interactions that result in changes to Ca^{2+} homeostasis and alteration of synaptic function in these cells may provide insights into the pathology responsible for the cognitive impairment in AD (Ridley et al., 1988; Paolini and McKenzie, 1993). The findings reported here illustrate the role of nAChRs in DBB neurons and suggests that nAChRs are an important receptor target for mediating the effects of $A\beta_{1-42}$ that may have important implications in processes such as neurodegeneration and synaptic dysfunction.

Aβ Potentiates Repeated nAChR Mediated $[Ca^{2+}]_i$ Rises

$A\beta_{1-42}$ potentiated nicotine-mediated $[Ca^{2+}]_i$ rises in acutely dissociated DBB neurons and was associated with a rise in basal Ca^{2+}. Some studies have suggested that in certain preparations, $A\beta_{1-42}$ is capable of forming Ca^{2+} ion channels, however the majority of these studies have either been in membrane bilayer systems or have only demonstrated the presence of $A\beta_{1-42}$ insertion onto the plasma membrane (Arispe et al., 1993; Rhee et al., 1998; Kawahara et al., 2000). In DBB neurons focal application of $A\beta_{1-42}$ did not cause any observable change in intracellular $[Ca^{2+}]_i$. Previous studies have demonstrated that $A\beta_{1-42}$ is capable of modulating activity of voltage gated Ca^{2+} channels (Price et al., 1998; Ramsden et al., 2002). The potentiation of nicotine-mediated $[Ca^{2+}]_i$

rises in acutely dissociated DBB neurons was not mediated by $A\beta_{1-42}$ effects on voltage-gated Ca^{2+} channels as $[Ca^{2+}]_i$ rises evoked by focal application of KCl were not potentiated by $A\beta_{1-42}$. Both extracellular and intracellular sources of Ca^{2+} contributed to nicotine-mediated $[Ca^{2+}]_i$ rises. Pharmacological characterization of the nicotine-induced Ca^{2+} rises revealed that both $\alpha7$ and $\alpha4\beta2$ nAChR subtypes mediated Ca^{2+} entry, however, there was a persistent component of Ca^{2+} rise that was not blocked by nAChR antagonists. The distribution of $\alpha7$ and $\alpha4$ nAChR subunits on DBB neurons was heterogeneous, which corresponded with the pharmacological data, with some cells displaying co-localization of the subunits on the same cell whereas others displayed labeling for either the $\alpha7$ or the $\alpha4$-subunit. These results are in line with previous studies examining nicotinic modulation of DBB neuron properties (Henderson et al., 2005). Additionally, anatomical evidence suggests that there are collateral ACh terminals from cholinergic neurons projecting to the hippocampus that mediate synapses onto DBB neurons (Henderson et al., 2001).

Further examination of the nicotine induced $[Ca^{2+}]_i$ rise revealed that emptying of intracellular Ca^{2+} stores by inhibiting the SERCA pump, which is responsible for filling of Ca^{-2+} stores, inhibits nicotine mediated $[Ca^{2+}]_i$ rises. The nicotine response could also be inhibited by xestospongin, an IP3 receptor blocker. This antagonist was previously used to demonstrate that $\alpha7$ nAChRs were coupled to IP3-sensitive stores, although given the data that xestospongin may also serve to inhibit SERCA pumps, this conclusion should be viewed with caution (De Smet et al., 1999; Castonguay and Robitaille, 2002; Dajas-Bailador et al., 2002; Solovyova et al., 2002).

$A\beta_{1-42}$ was also able to modulate Ca^{2+}-induced Ca^{2+} entry. Repeated application of caffeine to DBB neurons activated intracellular Ca^{2+} stores that could be inhibited by TG, which has been previously reported (Murchison and Griffith, 1999). In the presence of $A\beta_{1-42}$, the release of Ca^{2+} from these caffeine-sensitive stores were temporarily potentiated. This suggests that $A\beta_{1-42}$ was overfilling these caffeine-sensitive stores to the point where caffeine-sensitive intracellular stores were unable to buffer $[Ca^{2+}]_i$. However, unlike in the case of repeated nicotine-activation of intracellular Ca^{2+} stores, caffeine does not allow extracellular Ca^{2+} entry and thus there was no refilling of caffeine-sensitive stores. With no exogenous source of Ca^{2+} to replenish caffeine sensitive stores, the caffeine induced Ca^{2+} rise did not remain potentiated as with nicotine-evoked Ca^{2+} responses. It appears somewhat paradoxical that $A\beta_{1-42}$ was able to potentiate caffeine mediated Ca^{2+} rises, given that it could not potentiate Ca^{2+} rises mediated by KCl activation of voltage-gated Ca^{2+} channels. However, previous studies examining voltage-gated Ca^{2+}channels in DBB neurons revealed that Ca^{2+} entry via these channels is significantly buffered by the ER which prevents Ca^{2+} from activating Ca^{2+} release channels on intracellular Ca^{2+}stores (Murchison and Griffith, 1998).

$A\beta_{1-42}$ potentiation of nicotine-evoked $[Ca^{2+}]_i$ was mediated by its actions on TG sensitive intracellular Ca^{2+}stores. Blockade of these stores by CGP-37157, an inhibitory of the mitochondrial Na^+/Ca^{2+} exchanger after $A\beta_{1-42}$ potentiation of nicotine-mediated Ca^{2+} resulted in the blockade of the $A\beta_{1-42}$ potentiation. The decrease in the potentiation by Na^+/Ca^{2+} exchanger blockade was of similar magnitude to that observed with TG inhibition of the SERCA ATPase, which suggests that most of $A\beta_{1-42}$ potentiation of intracellular Ca^{2+} stores originates from overfilling of the TG-sensitive stores by

mitochondria. Thus we propose that $A\beta_{1-42}$ acts on mitochondria by causing the mitochondria to overfill with Ca^{2+}, which results in the excess release of Ca^{2+} via the Na^{+}/Ca^{2+} exchanger, which, in turn is taken up into the TG-sensitive stores by the action of the SERCA pumps (Figure 4-1). It has been demonstrated that mitochondria positioned close to these ER stores are responsible for buffering and refilling intracellular Ca^{2+} stores (Rizzuto et al., 1998). These observations are in line with previous studies which have demonstrated that blocking the Na^{+}/Ca^{2+} exchanger impairs Ca^{2+} release and that the Na^{+}/Ca^{2+} exchanger is localized in close proximity to SERCA pumps on the TG-sensitive stores (Arnaudeau et al., 2001; Csordas and Hajnoczky, 2001).

In this portion of experimental studies we have demonstrated that $A\beta_{1-42}$ alters Ca^{2+} homeostasis in DBB neurons via its effects on mitochondria (Figure 4-1). The effect of $A\beta_{1-42}$ on these mitochondria may be due to $A\beta_{1-42}$ interaction with a surface receptor such as the P75 or RAGE receptor (Yan et al., 1996; Yaar et al., 1997) or occur in a two step process with $A\beta_{1-42}$ entering the neuron by endocytosis, perhaps via the $\alpha 7$ nAChR (Wang et al., 2002) and then binding to an intracellular receptor. Modulation of mitochondrial Ca^{2+} is important in the regulation of neuronal apoptosis. $A\beta$ modulation of mitochondria by $A\beta_{1-42}$ has previously been shown to potentiate glutamate excitotoxicity and to trigger an apoptotic pathway involving the ER and caspases associated with this cellular organelle (Mattson et al., 1992; Nakagawa et al., 2000). Additionally, $A\beta_{1-42}$ modulation of the mitochondrial enzyme, $A\beta$-binding alcohol dehydrogenase, has been shown to impair the function of this enzyme in AD patients and to increase oxidative stress in AD transgenic mice (Lustbader et al., 2004).

Aβ Modulates Synaptic Transmission in Cholinergic DBB Neurons

In the second part of my project, the effect of $A\beta_{1-42}$ on spontaneous synaptic transmission in cholinergic neurons of the DBB was examined. Recordings of glutamate-mediated mEPSCs from fluorescently labeled cholinergic neurons in the DBB were examined. Application of $A\beta_{1-42}$ revealed cholinergic neurons of the DBB respond to $A\beta_{1-42}$ in two different ways. In the majority of neurons, $A\beta_{1-42}$ increased the frequency of mEPSCs. In a second population of neurons, $A\beta_{1-42}$ decreased the frequency of mEPSCs. In both groups of Aβ-responsive cells, $A\beta_{1-42}$ did not significantly change kinetic parameters such as rise time, decay time or amplitude, suggesting that $A\beta_{1-42}$ modulation of synaptic activity occurs via a presynaptic effect (Figure 4-2).

The increase in mEPSC frequency induced by $A\beta_{1-42}$ could be blocked in the presence of DHβE, a non-α7 nAChR antagonist, suggesting that $A\beta_{1-42}$ mediates this effect on synaptic transmission via a non-α7, α4β2 mechanism as the dose of DHβE used is in the low enough range to affect α4β2 nAChRs and not the other subtypes (Sharples and Wonnacott, 2001; Figure 4-2A). Previous electrophysiological studies examining non-α7 and α7 nAChR-$A\beta_{1-42}$ effects have been performed on expression systems and acutely dissociated neurons, and thus unable to assess the effect of modulation of synaptic activity. This study is the first to demonstrate that $A\beta_{1-42}$ is capable of altering spontaneous synaptic activity. As the basal forebrain plays an important role in learning and memory, alteration of synaptic function would interfere with cognition (Ridley et al., 1988; Paolini and McKenzie, 1993). Based upon this electrophysiology data, $A\beta_{1-42}$ can increase glutamate neurotransmission in a population of cholinergic neurons, while decreasing glutamate neurotransmission in another population of cholinergic neurons.

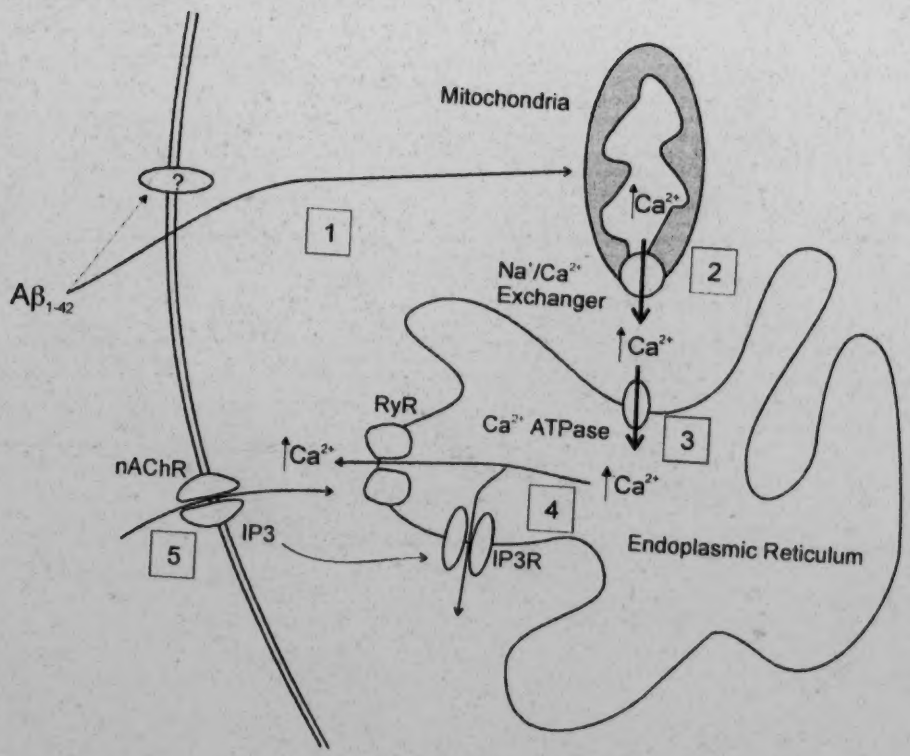
Although this would lead to an increase of ACh release from some cholinergic neurons, the increase in ACh would not necessarily be able to compensate for the loss of cholinergic tone from $A\beta_{1-42}$ inhibition of the other population of cholinergic neurons, as these neurons would synapse on different hippocampal neurons. Thus alteration of synaptic function in cholinergic neurons of the DBB by soluble $A\beta_{1-42}$ may provide the pathological basis for the mild cognitive dysfunction observed at the beginning of AD prior to the deposition of $A\beta_{1-42}$ plaques.

Application of other nicotinic agonists onto cholinergic neurons demonstrated that both nicotine and acetylcholine were able to modulate synaptic transmission in a manner similar to that observed with $A\beta_{1-42}$. This modulation is presynaptic in nature as only increases in mEPSC frequency were observed in this group of cells. It has been previously reported that nAChRs mediate post-synaptic actions, although these studies did not specifically rule out pre-synaptic modulation. One study examined the effect of nicotine application on acutely dissociated DBB neurons and whereas another examined single channel currents, thus these studies were not specifically looking for presynaptic modulation of neurotransmitter release (Fu and Jhamandas, 2003; Henderson et al., 2005).

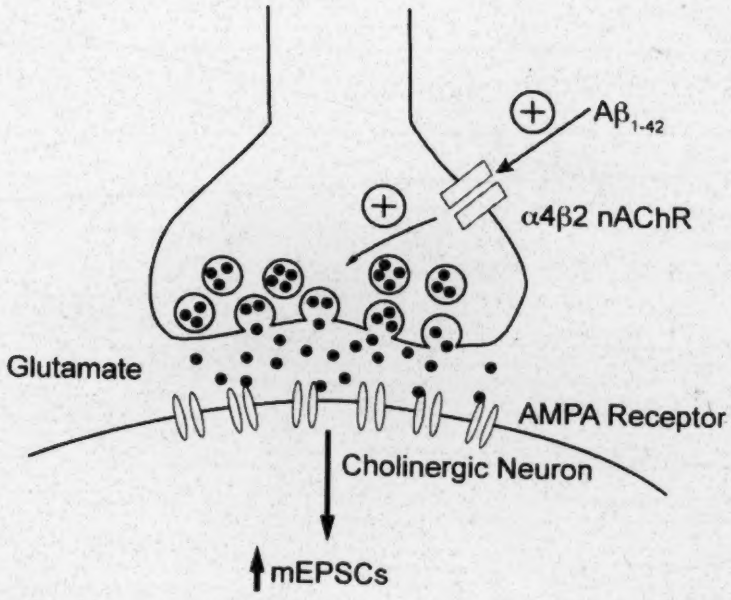
Future Experiments and Conclusions

In my thesis, two separate $A\beta_{1-42}$ effects were demonstrated. Firstly, $A\beta_{1-42}$ was shown to alter and possibly disrupt Ca^{2+} homeostasis via mediating overfilling of SERCA stores through the release of Ca^{2+} via the Na^{+}/Ca^{2+} exchanger. Secondly, $A\beta_{1-42}$ was able to modulate excitatory synaptic function in the basal forebrain. With regards to the first set of experiments, significant questions remain as to the role of experiments measuring

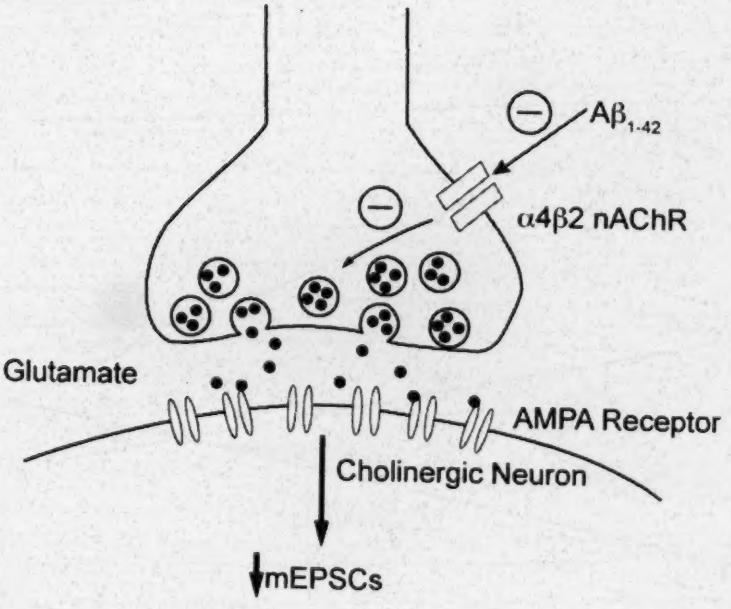
mitochondrial membrane potential and mitochondrial Ca^{2+} may provide more information about the effect of $A\beta_{1-42}$ on mitochondria and the relationship of this to ER stress which has been postulated to be a major player in neuronal death evoked by Aβ and other similarly misfolded proteins. Further experiments examining mitochondria will provide additional insights into how $A\beta_{1-42}$ may overfill mitochondria Ca^{2+}. Examining potential targets through which $A\beta_{1-42}$ mediates a decrease in mEPSC frequency also need to be performed. Furthermore, pharmacological characterization of the nAChR responses in the DBB needs to be examined as targeting specific subtypes of nAChRs may become important to AD therapy.


Mitochondria
?
1
$A\beta_{1-42}$
Ca^{2+}
2
Na^+/Ca^{2+} Exchanger
Ca^{2+}
RyR
Ca^{2+} ATPase
3
Ca^{2+}
nAChR
Ca^{2+}
4
5
IP3
IP3R
Endoplasmic Reticulum

A.


Aβ1-42
α4β2 nAChR
Glutamate
AMPA Receptor
Cholinergic Neuron
mEPSCs

B.


Aβ1-42
α4β2 nAChR
Glutamate
AMPA Receptor
Cholinergic Neuron
mEPSCs

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